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(54) Title: IMMUNOGLOBULIN VARIANTS AND USES THEREOF

(57) Abstract: The invention provides humanized and chimeric anti-CD20 antibodies for treatment of CD20 positive malignancies and autoimmune diseases.

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IMMUNOGLOBULIN VARIANTS AND USES THEREOF

FIELD OF THE INVENTION

10   The invention relates to anti-CD20 antibodies and their use in the treatment of B-cell related

diseases.

BACKGROUND OF THE INVENTION

Lymphocytes are one of several populations of white blood cells; they specifically recognize and

15   respond to foreign antigen. The three major classes of lymphocytes are B lymphocytes (B cells), T

lymphocytes (T cells) and natural killer (NK) cells. B lymphocytes are the cells responsible for antibody

production and provide humoral immunity. B cells mature within the bone marrow and leave the marrow

expressing an antigen-binding antibody on their cell surface. When a naive B cell first encounters the

antigen for which its membrane-bound antibody is specific, the cell begins to divide rapidly and its progeny

20   differentiate into memory B cells and effector cells called "plasma cells". Memory B cells have a longer life

span and continue to express membrane-bound antibody with the same specificity as the original parent cell.

Plasma cells do not produce membrane-bound antibody but instead produce secreted form of the antibody.

Secreted antibodies are the major effector molecules of humoral immunity.

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, Bp35) is a

25   hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and

mature B lymphocytes (Valentine *et al. J. Biol. Chem.* 264(19):11282-11287 (1989); and Einfeld *et al.*

EMBO J. 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's

lymphomas (NHL) (Anderson *et al. Blood* 63(6):1424-1433 (1984)), but is not found on hematopoietic stem

cells, pro-B cells, normal plasma cells or other normal tissues (Tedder *et al. J. Immunol.* 135(2):973-979

30   (1985)). CD20 is thought to regulate an early step(s) in the activation process for cell cycle initiation and

differentiation (Tedder *et al., supra*) and possibly functions as a calcium ion channel (Tedder *et al. J. Cell.*

Biochem. 14D:195 (1990)).

Given the expression of CD20 in B cell lymphomas, this antigen has been a useful therapeutic

target to treat such lymphomas. There are more than 300,000 people in the United States with B-cell NHL

35   and more than 56,000 new cases are diagnosed each year. For example, the rituximab (RITUXAN®)

antibody which is a genetically engineered chimeric murine/human monoclonal antibody directed against

human CD20 antigen (commercially available from Genentech, Inc., South San Francisco, California, U.S.)

is used for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B cell

non-Hodgkin's lymphoma. Rituximab is the antibody referred to as "C2B8" in US Patent No. 5,736,137

40   issued April 7, 1998 (Anderson *et al.*) and in US Pat No. 5,776,456. *In vitro* mechanism of action studies

have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through

complement-dependent cytotoxicity (CDC) (Reff *et al. Blood* 83(2):435-445 (1994)). Additionally, it has

significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). *In vivo* preclinical

studies have shown that RITUXAN® depletes B cells from the peripheral blood, lymph nodes, and bone

45   marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff *et al.*

5   *Blood* 83(2):435-445 (1994)). Other anti-CD20 antibodies indicated for the treatment of NHL include the

murine antibody Zevalin™ which is linked to the radioisotope, Yttrium-90 (IDEC Pharmaceuticals, San

Diego, CA), Bexxar™ which is a another fully murine antibody conjugated to I-131 (Corixa, WA).

A major limitation in the use of murine antibodies in human therapy is the human anti-mouse

antibody (HAMA) response (see, e.g., Miller, R.A. et al. "Monoclonal antibody therapeutic trials in seven

10   patients with T-cell lymphoma" Blood, 62:988-995, 1983; and Schroff, R.W., et al. "Human anti-murine

immunoglobulin response in patients receiving monoclonal antibody therapy" Cancer Res. , 45:879-885,

1985). Even chimeric molecules, where the variable (V) domains of rodent antibodies are fused to human

constant (C) regions, are still capable of eliciting a significant immune response (HACA, human anti-

chimeric antibody) (Neuberger et al. Nature (Lond.), 314:268-270, 1985). A powerful approach to

15   overcome these limitations in the clinical use of monoclonal antibodies is "humanization" of the murine

antibody or antibody from a non-human species (Jones *et al.* Nature (Lond), 321:522-525, 1986; Riechman

et al., Nature (Lond), 332:323-327, 1988).

Thus, it is beneficial to produce therapeutic antibodies to the CD20 antigen that create minimal or

no antigenicity when administered to patients, especially for chronic treatment. The present invention

20   satisfies this and other needs. The present invention provides anti-CD20 antibodies that overcome the

limitations of current therapeutic compositions as well as offer additional advantages that will be apparent

from the detailed description below.

SUMMARY OF THE INVENTION

25   The present invention provides CD20 binding antibodies or functional fragments thereof, and their

use in the treatment of B-cell associated diseases. These antibodies are monoclonal antibodies. In specific

embodiments, the antibodies that bind CD20 are humanized or chimeric. The humanized 2H7 variants

include those that have amino acid substitutions in the FR and affinity maturation variants with changes in

the grafted CDRs. The substituted amino acids in the CDR or FR are not limited to those present in the

30   donor or recipient antibody. In other embodiments, the anti-CD20 antibodies of the invention further

comprise changes in amino acid residues in the Fc region that lead to improved effector function including

enhanced CDC and/or ADCC function and B-cell killing (also referred to herein as B-cell depletion). Other

anti-CD20 antibodies of the invention include those having specific changes that improve stability. In a

specific embodiment, the humanized 2H7 variants with increased stability are as described in example 6

35   below. Fucose deficient variants having improved ADCC function in vivo are also provided. In one

embodiment, the chimeric anti-CD20 antibody has murine V regions and human C region. One such

specific chimeric anti-CD20 antibody is Rituxan® (Rituximab®; Genentech, Inc.).

In a preferred embodiment of all of the antibody compositions and methods of use of this invention,

the humanized CD20 binding antibody is 2H7.v16 having the light and heavy chain amino acid sequence of

40   SEQ ID NO. 21 and 22, respectively, as shown in FIG. 6 and FIG. 7. When referring to the polypeptide

sequences in Figures 6, 7 and 8, it should be understood that the first 19 or so amino acids that form the

secretory signal sequence are not present in the mature polypeptide. The V region of all other variants based

on version 16 will have the amino acid sequences of v16 except at the positions of amino acid substitutions

5   which are indicated in the disclosure. Unless otherwise indicated, the 2H7 variants will have the same L

chain as that of v16.

The invention provides a humanized antibody that binds human CD20, or an antigen-binding

fragment thereof, wherein the antibody is effective to deplete primate B cells in vivo, the antibody

comprising in the H chain Variable region (V_H) at least a CDR3 sequence of SEQ ID NO. 12 from an anti-

10   human CD20 antibody and substantially the human consensus framework (FR) residues of human heavy

chain subgroup III (V_HIII). In one embodiment, the primate B cells are from human and Cynomolgus

monkey. In one embodiment, the antibody further comprises the H chain CDR1 sequence of SEQ ID NO.

10 and CDR2 sequence of SEQ ID NO. 11. In another embodiment, the preceding antibody comprises the L

chain CDR1 sequence of SEQ ID NO. 4, CDR2 sequence of SEQ ID NO. 5, CDR3 sequence of SEQ ID

15   NO. 6 with substantially the human consensus framework (FR) residues of human light chain κ subgroup I

(VκI). In a preferred embodiment, the FR region in V_L has a donor antibody residue at position 46; in a

specific embodiment, FR2 in V_L has an amino acid substitution of leuL46pro (Leu in the human κI

consensus sequence changed to pro which is present in the corresponding position in m2H7).

The VH region further comprises a donor antibody residue at at least amino acid positions 49, 71 and 73 in

20   the framework. In one embodiment, in the V_H, the following FR positions in the human heavy chain

subgroup III are substituted: AlaH49Gly in FR2; ArgH71Val and AsnH73Lys in FR3. In other

embodiments, the CDR regions in the humanized antibody further comprise amino acid substitutions where

the residues are neither from donor nor recipient antibody.

The antibody of the preceding embodiments can comprise the V_H sequence of SEQ ID NO.8 of

25   v16, as shown in FIG. 1B. In a further embodiment of the preceding, the antibody further comprises the V_L

sequence of SEQ ID NO.2 of v16, as shown in FIG. 1A.

In other embodiments, the humanized antibody is 2H7.v31 having the light and heavy chain amino

acid sequence of SEQ ID NO. 2 and 23, respectively, as shown in FIG. 6 and FIG. 8; 2H7.v31 having the

heavy chain amino acid sequence of SEQ ID NO. 23 as shown in FIG. 8; 2H7.v96 with the amino acid

30   substitutions of D56A and N100A in the H chain and S92A in the L chain of v16.

In separate embodiments, the antibody of any of the preceding embodiments further comprises at

least one amino acid substitution in the Fc region that improves ADCC and/or CDC activity over the original

or parent antibody from which it was derived, v.16 being the parent antibody being compared to in most

cases, and Rituxan in other cases. One such antibody with improved activity comprises the triple Alanine

35   substitution of S298A/E333A/K334A in the Fc region. One antibody having S298A/E333A/K334A

substitution is 2H7.v31 having the heavy chain amino acid sequence of SEQ ID NO. 23. Antibody

2H7.v114 and 2H7.v115 show at least 10-fold improved ADCC activity as compared to Rituxan.

In another embodiment, the antibody further comprises at least one amino acid substitution in the

Fc region that decreases CDC activity as compared to the parent antibody from which it was derived which

40   is v16 in most cases. One such antibody with decreased CDC activity as compared to v16 comprises at least

the substitution K322A in the H chain. The comparison of ADCC and CDC activity can be assayed as

described in the examples.

5   In a preferred embodiment, the antibodies of the invention are full length antibodies wherein the V_H

region is joined to a human IgG heavy chain constant region. In preferred embodiments, the IgG is human

IgG1 or IgG3.

           In one embodiment, the CD20 binding antibody is conjugated to a cytotoxic agent. In preferred

embodiments the cytotoxic agent is a toxin or a radioactive isotope.

10   In one embodiment, the antibodies of the invention for use in therapeutic or diagnostic purposes are

produced in CHO cells.

           Also provided is a composition comprising an antibody of any one of the preceding embodiments,

and a carrier. In one embodiment, the carrier is a pharmaceutically acceptable carrier. These compositions

can be provided in an article of manufacture or a kit.

15   The invention also provided a liquid formulation comprising a humanized 2H7 antibody at

20mg/mL antibody, 10mM histidine sulfate pH5.8, 60mg/ml sucrose (6%), 0.2 mg/ml polysorbate 20

(0.02%).

           The invention also provides an isolated nucleic acid that encodes any of the antibodies disclosed

herein, including an expression vector for expressing the antibody.

20   Another aspect of the invention are host cells comprising the preceding nucleic acids, and host cells

that produce the antibody. In a preferred embodiment of the latter, the host cell is a CHO cell. A method of

producing these antibodies is provided, the method comprising culturing the host cell that produces the

antibody and recovering the antibody from the cell culture.

           Yet another aspect of the invention is an article of manufacture comprising a container and a

25   composition contained therein, wherein the composition comprises an antibody of any of the preceding

embodiments. For use in treating NHL, the article of manufacture further comprises a package insert

indicating that the composition is used to treat non-Hodgkin's lymphoma.

           A further aspect of the invention is a method of inducing apoptosis in B cells in vivo, comprising

contacting B cells with the antibody of any of the preceding, thereby killing the B cells.

30   The invention also provides methods of treating the diseases disclosed herein by administration of a

CD20 binding antibody or functional fragment thereof, to a mammal such as a human patient suffering from

the disease. In any of the methods for treating an autoimmune disease or a CD20 positive cancer, in one

embodiment, the antibody is 2H7.v16 having the light and heavy chain amino acid sequence of SEQ ID NO.

21 and 22, respectively, as shown in FIG. 6 and FIG. 7. Thus, one embodiment is a method of treating a

35   CD20 positive cancer, comprising administering to a patient suffering from the cancer, a therapeutically

effective amount of a humanized CD20 binding antibody of the invention. In preferred embodiments, the

CD20 positive cancer is a B cell lymphoma or leukemia including non-Hodgkin's lymphoma (NHL) or

lymphocyte predominant Hodgkin's disease (LPHD), chronic lymphocytic leukemia (CLL) or SLL. In one

embodiment of the method of treating a B cell lymphoma or leukemia, the antibody is administered at a

40   dosage range of about 275-375mg/m². In additional embodiments, the treatment method further comprises

administering to the patient at least one chemotherapeutic agent, wherein for non-Hodgkin's lymphoma

(NHL), the chemotherapeutic agent is selected from the group consisting of doxorubicin, cyclophosphamide,

vincristine and prednisolone.

5   Also provided is a method of treating an autoimmune disease, comprising administering to a patient

suffering from the autoimmune disease, a therapeutically effective amount of the humanized CD20 binding

antibody of any one of the preceding claims. The autoimmune disease is selected from the group consisting

of rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's

disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic

10   thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA

nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome,

Sjorgen's syndrome and glomerulonephritis. Where the autoimmune disease is rheumatoid arthritis, the

antibody can be administered in conjunction with a second therapeutic agent which is preferably

methotrexate.

15   In these treatment methods, the CD20 binding antibodies can be administered alone or in

conjunction with a second therapeutic agent such as a second antibody, or a chemotherapeutic agent or an

immunosuppressive agent. The second antibody can be one that binds CD20 or a different B cell antigen, or

a NK or T cell antigen. In one embodiment, the second antibody is a radiolabeled anti-CD20 antibody. In

other embodiments, the CD20 binding antibody is conjugated to a cytotoxic agent including a toxin or a

20   radioactive isotope.

In another aspect, the invention provides a method of treating an autoimmune disease selected from

the group consisting of Dermatomyositis, Wegner's granulomatosis, ANCA, Aplastic anemia, Autoimmune

hemolytic anemia (AIHA), factor VIII deficiency, hemophilia A, Autoimmune neutropenia, Castleman's

syndrome, Goodpasture's syndrome, solid organ transplant rejection, graft versus host disease (GVHD), IgM

25   mediated, thrombotic thrombocytopenic purpura (TTP), Hashimoto's Thyroiditis, autoimmune hepatitis,

lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs. NSIP, Guillain-Barre

Syndrome, large vessel vasculitis, giant cell (Takayasu's) arteritis, medium vessel vasculitis, Kawasaki's

Disease, polyarteritis nodosa, comprising administering to a patient suffering from the disease, a

therapeutically effective amount of a CD20 binding antibody. In one embodiment of this method, the CD20

30   binding antibody is Rituxan®.

The invention also provides an isolated nucleic acid comprising the nucleotide sequence of SEQ ID

NO.: _ of the Cynomolgus monkey CD20 (shown in FIG. 19), or a degenerate variant of this sequence . One

embodiment is an isolated nucleic acid comprising a sequence that encodes a polypeptide with the amino

acid sequence of SEQ ID NO. _ (shown FIG. 20), or SEQ ID NO. _ (FIG. 20) with conservative amino acid

35   substitutions. Another embodiment is a vector comprising the preceding nucleic acid, including an

expression vector for expression in a host cell. Included as well is a host cell comprising the vector. Also

provided is an isolated polypeptide comprising the amino acid sequence [SEQ ID NO. _ ; FIG. 20] of the

Cynomolgus monkey CD20.

40   **BRIEF DESCRIPTION OF THE FIGURES**

FIG. 1A is a sequence alignment comparing the amino acid sequences of the light chain variable

domain (V_L) of each of murine 2H7 (SEQ ID NO. 1), humanized 2H7. v16 variant (SEQ ID NO. 2), and

human kappa light chain subgroup I (SEQ ID NO. 3). The CDRs of V_L of 2H7 and hu2H7.v16 are as

follows: CDR1 (SEQ ID NO.4), CDR2 (SEQ ID NO.5), and CDR3 (SEQ ID NO.6).

5   FIG. 1B is a sequence alignment which compares the V_H sequences of murine 2H7 (SEQ ID NO.

7), humanized 2H7.v16 variant (SEQ ID NO. 8), and the human consensus sequence of heavy chain

subgroup III (SEQ ID NO. 9). The CDRs of V_H of 2H7 and hu2H7.v16 are as follow: CDR1 (SEQ ID

NO.10), CDR2 (SEQ ID NO.11), and CDR3 (SEQ ID NO.12).

          In FIG. 1A and FIG. 1B, the CDR1, CDR2 and CDR3 in each chain are enclosed within brackets,

10   flanked by the framework regions, FR1-FR4, as indicated. 2H7 refers to the murine 2H7 antibody. The

asterisks in between two rows of sequences indicate the positions that are different between the two

sequences. Residue numbering is according to Kabat et al., Sequences of Immunological Interest. 5th Ed.

Public Health Service, National Institutes of Health, Bethesda, Md. (1991), with insertions shown as a, b, c,

d, and e.

15   FIG. 2 shows the sequence of phagemid pVX4 (SEQ ID NO.13) used for construction of 2H7 Fab

plasmids (see Example 1) as well as the amino acid sequences of the L chain (SEQ ID NO.14) and H chain

(SEQ ID NO.15) of the Fab for the CDR-grafted anti-IFN-α humanized antibody.

          FIG. 3 shows the sequence of the expression plasmid which encodes the chimeric 2H7.v6.8 Fab

(SEQ ID NO.16). The amino acid sequences of the L chain (SEQ ID NO.17) and H chain (SEQ ID NO.18)

20   are shown.

          FIG. 4 shows the sequence of the plasmid pDR1 (SEQ ID NO.19; 5391 bp) for expression of

immunoglobulin light chains as described in Example 1. pDR1 contains sequences encoding an irrelevant

antibody, the light chain of a humanized anti-CD3 antibody (Shalaby et al., J. Exp. Med. 175: 217-225

(1992)), the start and stop codons for which are indicated in bold and underlined.

25   FIG. 5 shows the sequence of plasmid pDR2 (SEQ ID NO.20; 6135 bp) for expression of

immunoglobulin heavy chains as described in Example 1. pDR2 contains sequences encoding an irrelevant

antibody, the heavy chain of a humanized anti-CD3 antibody (Shalaby et al., *supra*), the start and stop

codons for which are indicated in bold and underlined.

          FIG. 6 shows the amino acid sequence of the 2H7.v16 complete L chain (SEQ ID NO.21). The first

30   19 amino acids before DIQ are the secretory signal sequence not present in the mature polypeptide chain.

          FIG. 7 shows the amino acid sequence of the 2H7.v16 complete H chain (SEQ ID NO.22). The

first 19 amino acids before EVQ before are the secretory signal sequence not present in the mature

polypeptide chain. Aligning the V_H sequence in FIG. 1B (SEQ ID NO. 8) with the complete H chain

sequence, the human γ1 constant region is from amino acid position 114-471 in SEQ ID NO. 22.

35   FIG. 8 shows the amino acid sequence of the 2H7.v31 complete H chain (SEQ ID NO.23). The

first 19 amino acids before EVQ before are the secretory signal sequence not present in the mature

polypeptide chain. The L chain is the same as for 2H7.v16 (see FIG. 6).

          FIG. 9 shows the relative stability of 2H7.v16 and 2H7.v73 IgG variants. Assay results were

normalized to the values prior to incubation and reported as percent remaining after incubation.

40   FIG. 10 is a flow chart summarizing the amino acid changes from the murine 2H7 to a subset of

humanized versions up to v75.

          FIG. 11 is a summary of mean absolute B-cell count [CD3-/CD40+] in all groups (2H7 study and

Rituxan study combined), as described in Example 10.

5   FIG. 12 shows the results of a representative ADCC assay on fucose deficient 2H7 variants as described in Example 11.

        FIG. 13 shows the results of the Annexin V staining plotted as a function of antibody concentration. Ramos cells were treated with an irrelevant IgG1 control antibody (Herceptin®; circles), Rituximab (squares), or rhuMAb 2H7.v16 (triangles) in the presence of a crosslinking secondary antibody and were

10   analyzed by FACS. Figures 13-15 are described in Example 13.

        FIG. 14 shows the results of the Annexin V and propidium iodide double-staining are plotted as a function of antibody concentration. Ramos cells were treated with an irrelevant IgG1 control antibody (Herceptin®; circles), Rituximab (squares), or rhuMAb 2H7.v16 (triangles) in the presence of a crosslinking secondary antibody and were analyzed by FACS.

15   FIG. 15 shows the counts (per 10 s) of live, unstained cells are plotted as a function of antibody concentration. Ramos cells were treated with an irrelevant IgG1 control antibody (Herceptin®; circles), Rituximab (squares), or rhuMAb 2H7.v16 (triangles) in the presence of a crosslinking secondary antibody and were analyzed by FACS.

        FIGs. 16, 17, 18 show inhibition of Raji cell tumor growth in nude mice, as described in Example

20   14. Animals were treated weekly (as indicated by vertical arrows; n=8 mice per group) for 6 weeks with PBS (control) or with Rituxan® or rhuMAb 2H7.v16 at 5 mg/kg (FIG. 16), 0.5 mg/kg (FIG. 17), or 0.05 mg/kg (FIG. 18).

        FIG. 19 shows the nucleotide (SEQ ID NO. _) and amino acid (SEQ ID NO. _) sequences of Cynomolgus monkey CD20, as described in Example 15.

25   FIG. 20 shows the amino acid sequence for cynomolgus monkey CD20. Residues that differ from human CD20 are underlined and the human residues are indicated directly below the monkey residue. The putative extracellular domain of the monkey CD20 is in bold type.

        FIG. 21 shows the results of Cynomolgus monkey cells expressing CD20 binding to hu2H7.v16, .v31, and Rituxan, as described in Example 15. The antibodies were assayed for the ability to bind and

30   displace FITC-conjugated murine 2H7 binding to cynomolgus CD20.

        FIG. 22 shows dose escalation schema for rheumatoid arthritis phase I/II clinical trial.

        FIG. 23 shows the vector for expression of 2H7.v16 in CHO cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

35   The "CD20" antigen is a non-glycosylated, transmembrane phosphoprotein with a molecular weight of approximately 35 kD that is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation; it is not found on human stem cells, lymphoid progenitor cells or normal plasma cells. CD20 is present on both normal B cells as well as malignant B cells. Other names for CD20 in the literature

40   include "B-lymphocyte-restricted differentiation antigen" and "Bp35". The CD20 antigen is described in, for example, Clark and Ledbetter, *Adv. Can. Res.* 52:81-149 (1989) and Valentine *et al. J. Biol. Chem.* 264(19):11282-11287 (1989).

5   The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity or function.

       The biological activity of the CD20 binding and humanized CD20 binding antibodies of the invention will include at least binding of the antibody to human CD20, more preferably binding to human

10   and other primate CD20 (including cynomolgus monkey, rhesus monkey, chimpanzees). The antibodies would bind CD20 with a K_d value of no higher than 1×10^{-8}, preferably a K_d value no higher than about 1×10^{-9}, and be able to kill or deplete B cells in vivo, preferably by at least 20% when compared to the appropriate negative control which is not treated with such an antibody. B cell depletion can be a result of one or more of ADCC, CDC, apoptosis, or other mechanism. In some embodiments of disease treatment

15   herein, specific effector functions or mechanisms may be desired over others and certain variants of the humanized 2H7 are preferred to achieve those biological functions, such as ADCC.

       "Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')$_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from

20   antibody fragments.

       "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and - binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer

25   antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

       The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical

30   except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a

35   substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described

40   in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

       "Functional fragments" of the CD20 binding antibodies of the invention are those fragments that retain binding to CD20 with substantially the same affinity as the intact full length molecule from which

5   they are derived and show biological activity including depleting B cells as measured by in vitro or in vivo
       assays such as those described herein.

              The term "variable" refers to the fact that certain segments of the variable domains differ
       extensively in sequence among antibodies. The V domain mediates antigen binding and define specificity of
       a particular antibody for its particular antigen. However, the variability is not evenly distributed across the
10   110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches
       called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability
       called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy
       and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three
       hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet
15   structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with
       the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of
       antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service,
       National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in
       binding an antibody to an antigen, but exhibit various effector functions, such as participation of the
20   antibody in antibody dependent cellular cytotoxicity (ADCC).

              The term "hypervariable region" when used herein refers to the amino acid residues of an antibody
       which are responsible for antigen-binding. The hypervariable region generally comprises amino acid
       residues from a "complementarity determining region" or "CDR" (e.g. around about residues 24-34 (L1),
       50-56 (L2) and 89-97 (L3) in the V_L, and around about 31-35B (H1), 50-65 (H2) and 95-102 (H3) in the V_H
25   (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National
       Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues
       26-32 (L1), 50-52 (L2) and 91-96 (L3) in the V_L, and 26-32 (H1), 52A-55 (H2) and 96-101 (H3) in the V_H
       (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)).

              As referred to herein, the "consensus sequence" or consensus V domain sequence is an artificial
30   sequence derived from a comparison of the amino acid sequences of known human immunoglobulin variable
       region sequences. Based on these comparisons, recombinant nucleic acid sequences encoding the V domain
       amino acids that are a consensus of the sequences derived from the human κ and the human H chain
       subgroup III V domains were prepared. The consensus V sequence does not have any known antibody
       binding specificity or affinity.
35   "Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical
       with or homologous to corresponding sequences in antibodies derived from a particular species or belonging
       to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous
       to corresponding sequences in antibodies derived from another species or belonging to another antibody
       class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological
40   activity (U.S. Patent No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)).
       Humanized antibody as used herein is a subset of chimeric antibodies.

              "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain
       minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are

5   human immunoglobulins (recipient or acceptor antibody) in which hypervariable region residues of the

recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as

mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some

instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding

non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the

10   recipient antibody or in the donor antibody. These modifications are made to further refine antibody

performance such as binding affinity. Generally, the humanized antibody will comprise substantially all of

at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops

correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those

of a human immunoglobulin sequence although the FR regions may include one or more amino acid

15   substitutions that improve binding affinity. The number of these amino acid substitutions in the FR are

typically no more than 6 in the H chain, and in the L chain, no more than 3. The humanized antibody

optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a

human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al.,*

Nature 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

20   Antibody "effector functions" refer to those biological activities attributable to the Fc region (a

native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the

antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent

cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis;

down regulation of cell surface receptors (*e.g.* B cell receptor); and B cell activation.

25   "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in

which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (*e.g.* Natural Killer (NK)

cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-

bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic

cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express

30   FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic

cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To

assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent

No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral

blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC

35   activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in

Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The

preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG

antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including

40   allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an

"activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that

differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an

immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor

FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see

5   review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim

10   *et al., J. Immunol.* 24:249 (1994)).

WO00/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. The content of that patent publication is specifically incorporated herein by reference. See, also, Shields *et al. J. Biol. Chem.* 9(2): 6591-6604 (2001).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector

15   functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, *e.g.* from blood.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence

20   of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed.

Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q

25   binding capability are described in US patent No. 6,194,551B1 and WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

The N-glycosylation site in IgG is at Asn297 in the CH2 domain. The present invention also provides compositions of a CD20-binding, humanized antibody having a Fc region, wherein about 80-100%

30   (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to FcγRIIIA(F158), which is not as effective as FcγRIIIA (V158) in interacting with human IgG. Thus, the compositions herein are anticipated to be superior to previously described anti-CD20 antibody compositions, especially for therapy of human

35   patients who express FcγRIIIA (F158). FcγRIIIA (F158) is more common than FcγRIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher *et al. Blood* 94:4220 (1999). The present application further demonstrates the synergistic increase in FcγRIII binding and/or ADCC function that results from combining the glycosylation variations herein with amino acid sequence modification(s) in the Fc region of the glycoprotein.

40   An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most

5   preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or

internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE

under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated

antibody includes the antibody *in situ* within recombinant cells since at least one component of the

antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared

10   by at least one purification step.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from

at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of

the antibody nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is

found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule

15   as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule

contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a

chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an

operably linked coding sequence in a particular host organism. The control sequences that are suitable for

20   prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site.

Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another

nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA

for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a

25   promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence;

or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate

translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and,

in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be

contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the

30   synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

"Vector" includes shuttle and expression vectors. Typically, the plasmid construct will also include

an origin of replication (*e.g.*, the ColE1 origin of replication) and a selectable marker (*e.g.*, ampicillin or

tetracycline resistance), for replication and selection, respectively, of the plasmids in bacteria. An

"expression vector" refers to a vector that contains the necessary control sequences or regulatory elements

35   for expression of the antibodies including antibody fragment of the invention, in bacterial or eukaryotic cells.

Suitable vectors are disclosed below.

The cell that produces a humanized CD20 binding antibody of the invention will include the

bacterial and eukaryotic host cells into which nucleic acid encoding the antibodies have been introduced.

Suitable host cells are disclosed below.

40   The word "label" when used herein refers to a detectable compound or composition which is

conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*,

radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical

alteration of a substrate compound or composition which is detectable.

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5   An "autoimmune disease" herein is a non-malignant disease or disorder arising from and directed

against an individual's own (self) antigens and/or tissues.

As used herein, "B cell depletion" refers to a reduction in B cell levels in an animal or human after drug or

antibody treatment, as compared to the B cell level before treatment. B cell levels are measurable using well

known assays such as those described in the Experimental Examples. B cell depletion can be complete or

10   partial. In one embodiment, the depletion of CD20 expressing B cells is at least 25%. Not to be limited by

any one mechanism, possible mechanisms of B-cell depletion include ADCC, CDC, apoptosis, modulation

of calcium flux or a combination of two or more of the preceding.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function

of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131},

15   I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial,

fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples

of chemotherapeutic agents include alkalyzing or alkylating agents such as thiotepa and cyclosphosphamide

(CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as

20   benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including

altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and

trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide,

estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin,

phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin,

25   fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin,

authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin,

chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin

(Adriamycin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid,

nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin,

30   streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-

fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine

analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as

ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine,

floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane,

35   testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as

frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil;

bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid;

gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine;

pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane;

40   sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2''-trichlorotriethylamine; urethan; vindesine;

dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C");

thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, NJ) and doxetaxel

(TAXOTERE®, Rhône-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine;

mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; platinum; etoposide (VP-

5   16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinblastine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example

10   tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; other chemotherapeutic agents such as prednisolone. Pharmaceutically acceptable salts, acids or derivatives of any of the above are included.

        "Treating" or "treatment" or "alleviation" refers to both therapeutic treatment and prophylactic or

15   preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. A subject is successfully "treated" for a CD20 positive cancer or an autoimmune disease if, after receiving a therapeutic amount of a CD20 binding antibody of the invention according to the methods of the present invention, the subject shows observable and/or measurable reduction in or absence of one or more signs and symptoms of the particular disease. For example, for cancer, reduction in the number

20   of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; increase in length of remission, and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. Reduction of the signs or symptoms of a disease may also be felt by the patient. Treatment can achieve a complete response,

25   defined as disappearance of all signs of cancer, or a partial response, wherein the size of the tumor is decreased, preferably by more than 50 percent, more preferably by 75%. A patient is also considered treated if the patient experiences stable disease. In a preferred embodiment, the cancer patients are still progression-free in the cancer after one year, preferably after 15 months. These parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar

30   to a physician of appropriate skill in the art.

        A "therapeutically effective amount" refers to an amount of an antibody or a drug effective to "treat" a disease or disorder in a subject. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and

35   preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See preceding definition of "treating".

        "Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is

40   cyclic in nature.

Compositions and Methods of the Invention

        The invention provides humanized antibodies that bind human CD20, and preferably other primate CD20 as well, comprising a H chain having at least one, preferably two or all of the H chain CDRs of a non-

5   human species anti-human CD20 antibody (donor antibody), and substantially all of the framework residues

of a human consensus antibody as the recipient antibody. The donor antibody can be from various non-

human species including mouse, rat, guinea pig, goat, rabbit, horse, primate but most frequently will be a

murine antibody. "Substantially all" in this context is meant that the recipient FR regions in the humanized

antibody may include one or more amino acid substitutions not originally present in the human consensus

10   FR sequence. These FR changes may comprise residues not found in the recipient or the donor antibody.

In one embodiment, the donor antibody is the murine 2H7 antibody, the V region including the

CDR and FR sequences of each of the H and L chains of which are shown in FIG. 1A and 1B. In a specific

embodiment, the residues for the human Fab framework correspond to the consensus sequence of human

Vκ subgroup I and of V_H subgroup III , these consensus sequences are shown in Figure 1A and Figure 1B,

15   respectively. The humanized 2H7 antibody of the invention will have at least one of the CDRs in the H

chain of the murine donor antibody. In one embodiment, the humanized 2H7 antibody that binds human

CD20 comprises the CDRs of both the H and L chains of the donor antibody.

In a full length antibody, the humanized CD20 binding antibody of the invention will comprise a

humanized V domain joined to a C domain of a human immunoglobulin. In a preferred embodiment, the H

20   chain C region is from human IgG, preferably IgG1 or IgG3. The L chain C domain is preferably from

human κ chain.

Unless indicated otherwise, a humanized 2H7 antibody version herein will have the V and C

domain sequences of 2H7.v16 L chain (FIG. 6, SEQ ID NO. 21) and H chain (FIG 7., SEQ ID NO. 22)

except at the positions of amino acid substitutions or changes indicated in the experimental examples below.

25   The humanized CD20 binding antibodies will bind at least human CD20 and preferably bind other

primate CD20 such as that of monkeys including cynomolgus and rhesus monkeys, and chimpanzees. The

sequence of the cynomolgus monkey CD20 is disclosed in Example 15 and Figure 19

The biological activity of the CD20 binding antibodies and humanized CD20 binding antibodies of

the invention will include at least binding of the antibody to human CD20, more preferably binding to

30   human and primate CD20 (including cynomolgus monkey, rhesus monkey, chimpanzees), with a K_d value of

no higher than 1×10^{-8}, preferably a K_d value no higher than about 1×10^{-9}, even more preferably a K_d value

no higher than about 1×10^{-10}, and be able to kill or deplete B cells in vitro or in vivo, preferably by at least

20% when compared to the baseline level or appropriate negative control which is not treated with such an

antibody.

35   The desired level of B cell depletion will depend on the disease. For the treatment of a CD20

positive cancer, it may be desirable to maximize the depletion of the B cells which are the target of the anti-

CD20 antibodies of the invention. Thus, for the treatment of a CD20 positive B cell neoplasm, it is desirable

that the B cell depletion be sufficient to at least prevent progression of the disease which can be assessed by

the physician of skill in the art, e.g., by monitoring tumor growth (size), proliferation of the cancerous cell

40   type, metastasis, other signs and symptoms of the particular cancer. Preferably, the B cell depletion is

sufficient to prevent progression of disease for at least 2 months, more preferably 3 months, even more

preferably 4 months, more preferably 5 months, even more preferably 6 or more months. In even more

preferred embodiments, the B cell depletion is sufficient to increase the time in remission by at least 6

months, more preferably 9 months, more preferably one year, more preferably 2 years, more preferably 3

5   years, even more preferably 5 or more years. In a most preferred embodiment, the B cell depletion is

sufficient to cure the disease. In preferred embodiments, the B cell depletion in a cancer patient is at least

about 75% and more preferably, 80%, 85%, 90%, 95% , 99% and even 100% of the baseline level before

treatment.

For treatment of an autoimmune disease, it may be desirable to modulate the extent of B cell

10   depletion depending on the disease and/or the severity of the condition in the individual patient, by adjusting

the dosage of CD20 binding antibody. Thus, B cell depletion can but does not have to be complete. Or,

total B cell depletion may be desired in initial treatment but in subsequent treatments, the dosage may be

adjusted to achieve only partial depletion. In one embodiment, the B cell depletion is at least 20%, i.e., 80%

or less of CD20 positive B cells remain as compared to the baseline level before treatment. In other

15   embodiments, B cell depletion is 25%, 30%, 40%, 50%, 60%, 70% or greater. Preferably, the B cell

depletion is sufficient to halt progression of the disease, more preferably to alleviate the signs and symptoms

of the particular disease under treatment, even more preferably to cure the disease.

The invention also provides bispecific CD20 binding antibodies wherein one arm of the antibody

has a humanized H and L chain of the humanized CD20 binding antibody of the invention, and the other arm

20   has V region binding specificity for a second antigen. In specific embodiments, the second antigen is

selected from the group consisting of CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

In comparison with Rituxan (rituximab), v16 exhibits about 2 to 5 fold increased ADCC potency ,

~3-4 fold decreased CDC than Rituxan.

25   **Antibody production**

Monoclonal antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.,*

Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is

30   immunized as described above to elicit lymphocytes that produce or are capable of producing antibodies that

will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized

in vitro. After immunization, lymphocytes are isolated and then fused with a myeloma cell line using a

suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal*

Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

35   The hybridoma cells thus prepared are seeded and grown in a suitable culture medium which

medium preferably contains one or more substances that inhibit the growth or survival of the unfused,

parental myeloma cells (also referred to as fusion partner). For example, if the parental myeloma cells lack

the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture

medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT

40   medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred fusion partner myeloma cells are those that fuse efficiently, support stable high-level

production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that

selects against the unfused parental cells. Preferred myeloma cell lines are murine myeloma lines, such as

those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution

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5   Center, San Diego, California USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); and Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

10   Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

          The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard
15   analysis described in Munson *et al., Anal. Biochem.*, 107:220 (1980).

          Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the
20   hybridoma cells may be grown *in vivo* as ascites tumors in an animal e.g, by i.p. injection of the cells into mice.

          The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography,
25   hydroxylapatite chromatography, gel electrophoresis, dialysis, etc.

          DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host
30   cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

35   In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks
40   *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

5   The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody
polypeptides, for example, by substituting human heavy chain and light chain constant domain (C_H and C_L)
sequences for the homologous murine sequences (U.S. Patent No. 4,816,567; and Morrison, *et al., Proc.
Natl Acad. Sci. USA*, 81:6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of
the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The non-
10   immunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are
substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric
bivalent antibody comprising one antigen-combining site having specificity for an antigen and another
antigen-combining site having specificity for a different antigen.

15   *Humanized antibodies*
         Methods for humanizing non-human antibodies have been described in the art. Preferably, a
humanized antibody has one or more amino acid residues introduced into it from a source which is non-
human. These non-human amino acid residues are often referred to as "import" residues, which are typically
taken from an "import" variable domain. Humanization can be essentially performed following the method
20   of Winter and co-workers (Jones *et al., Nature*, 321:522-525 (1986); Reichmann *et al., Nature*, 332:323-327
(1988); Verhoeyen *et al., Science*, 239:1534-1536 (1988)), by substituting hypervariable region sequences
for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are
chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable
domain has been substituted by the corresponding sequence from a non-human species. In practice,
25   humanized antibodies are typically human antibodies in which some hypervariable region residues and
possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.
         The choice of human variable domains, both light and heavy, to be used in making the humanized
antibodies is very important to reduce antigenicity and HAMA response (human anti-mouse antibody) when
the antibody is intended for human therapeutic use. According to the so-called "best-fit" method, the
30   sequence of the variable domain of a rodent antibody is screened against the entire library of known human
variable domain sequences. The human V domain sequence which is closest to that of the rodent is
identified and the human framework region (FR) within it accepted for the humanized antibody (Sims *et al.,
J. Immunol.*, 151:2296 (1993); Chothia *et al., J. Mol. Biol.*, 196:901 (1987)). Another method uses a
particular framework region derived from the consensus sequence of all human antibodies of a particular
35   subgroup of light or heavy chains. The same framework may be used for several different humanized
antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al., J. Immunol.*, 151:2623
(1993)).
         It is further important that antibodies be humanized with retention of high binding affinity for the
antigen and other favorable biological properties. To achieve this goal, according to a preferred method,
40   humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual
humanized products using three-dimensional models of the parental and humanized sequences. Three-
dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.
Computer programs are available which illustrate and display probable three-dimensional conformational
structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of

5   the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis

of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR

residues can be selected and combined from the recipient and import sequences so that the desired antibody

characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable

region residues are directly and most substantially involved in influencing antigen binding.

10   The humanized antibody may be an antibody fragment, such as a Fab, which is optionally

conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the

humanized antibody may be an full length antibody, such as an full length IgG1 antibody.

                    Human antibodies and phage display methodology

15   As an alternative to humanization, human antibodies can be generated. For example, it is now

possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full

repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it

has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in

chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

20   Transfer of the human germ-line immunoglobulin gene array into such germ-line mutant mice will result in

the production of human antibodies upon antigen challenge.   See, *e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad.*

Sci. USA, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggemann *et al.*, *Year in*

Immuno., 7:33 (1993); U.S. Patent Nos. 5,545,806, 5,569,825, 5,591,669 (all of GenPharm); 5,545,807; and

WO 97/17852.

25   Alternatively, phage display technology (McCafferty *et al.*, *Nature* 348:552-553 [1990]) can be

used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V)

domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes

are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as

M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the

30   filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the

functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting

those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be

performed in a variety of formats, reviewed in, *e.g.*, Johnson, Kevin S. and Chiswell, David J., *Current*

Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage

35   display. Clackson *et al.*, *Nature*, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies

from a small random combinatorial library of V genes derived from the spleens of immunized mice. A

repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array

of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks

et al., *J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al.*, *EMBO J.* 12:725-734 (1993). See, also, U.S.

40   Patent Nos. 5,565,332 and 5,573,905.

             As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see U.S.

Patents 5,567,610 and 5,229,275).

                                          19

Antibody fragments

In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.* , *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan *et al., Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')$_2$ fragments (Carter *et al.*, Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')$_2$ fragments can be isolated directly from recombinant host cell culture. Fab and F(ab')$_2$ fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Patent No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See *Antibody Engineering*, ed. Borrebaeck, supra. The antibody fragment may also be a "linear antibody", *e.g.*, as described in U.S. Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the CD20 protein. Other such antibodies may combine a CD20 binding site with a binding site for another protein. Alternatively, an anti-CD20 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), or NKG2D or other NK cell activating ligand, so as to focus and localize cellular defense mechanisms to the CD20-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express CD20. These antibodies possess a CD20-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab')$_2$ bispecific antibodies).

WO 96/16673 describes a bispecific anti-ErbB2/anti-FcγRIII antibody and U.S. Patent No. 5,837,234 discloses a bispecific anti-ErbB2/anti-FcγRI antibody. A bispecific anti-ErbB2/Fcα antibody is shown in WO98/02463. U.S. Patent No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

5   Methods for making bispecific antibodies are known in the art. Traditional production of full

length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain

pairs, where the two chains have different specificities (Millstein *et al.*, *Nature*, 305:537-539 (1983)).

Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas

(quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the

10   correct bispecific structure. Purification of the correct molecule, which is usually done by affinity

chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are

disclosed in WO 93/08829, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities

(antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. Preferably, the

15   fusion is with an Ig heavy chain constant domain, comprising at least part of the hinge, C_H2, and C_H3

regions. It is preferred to have the first heavy-chain constant region (C_H1) containing the site necessary for

light chain bonding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain

fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are

co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual

20   proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide

chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however,

possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector

when the expression of at least two polypeptide chains in equal ratios results in high yields or when the

ratios have no significant affect on the yield of the desired chain combination.

25   In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid

immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy

chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this

asymmetric structure facilitates the separation of the desired bispecific compound from unwanted

immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of

30   the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690.

For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in*

Enzymology, 121:210 (1986).

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair

of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered

35   from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain. In this

method, one or more small amino acid side chains from the interface of the first antibody molecule are

replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or

similar size to the large side chain(s) are created on the interface of the second antibody molecule by

replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a

40   mechanism for increasing the yield of the heterodimer over other unwanted end-products such as

homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the

antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for

example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and

5   for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al.,*
10   *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')$_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent, sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an
15   equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')$_2$ molecule. Each Fab' fragment was separately
20   secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from
25   recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers.
30   The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a V$_H$ connected to a V$_L$ by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V$_H$ and V$_L$ domains of one fragment are forced to pair with the complementary V$_L$ and V$_H$ domains of another fragment, thereby forming two antigen-binding sites. Another strategy for
35   making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

40   *Multivalent Antibodies*

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding

5   the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and

three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc

region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen

binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or

consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody

10   comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide

chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-

$(X1)_n$-VD2-$(X2)_n$-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one

polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For

instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-

15   CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two

(and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for

instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain

variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally,

further comprise a CL domain.

20

Other amino acid sequence modifications

Amino acid sequence modification(s) of the CD20 binding antibodies described herein are

contemplated. For example, it may be desirable to improve the binding affinity and/or other biological

properties of the antibody. Amino acid sequence variants of the anti-CD20 antibody are prepared by

25   introducing appropriate nucleotide changes into the anti-CD20 antibody nucleic acid, or by peptide

synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or

substitutions of, residues within the amino acid sequences of the anti-CD20 antibody. Any combination of

deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct

possesses the desired characteristics. The amino acid changes also may alter post-translational processes of

30   the anti-CD20 antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the anti-CD20 antibody that are

preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham

and Wells in *Science*, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.*,

charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino

35   acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with CD20 antigen.

Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by

introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing

an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be

predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or

40   random mutagenesis is conducted at the target codon or region and the expressed anti-CD20 antibody

variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length

from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions

of single or multiple amino acid residues. Examples of terminal insertions include an anti-CD20 antibody

5   with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional
       variants of the anti-CD20 antibody molecule include the fusion to the N- or C-terminus of the anti-CD20
       antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the
       antibody.

             Another type of variant is an amino acid substitution variant. These variants have at least one
10   amino acid residue in the anti-CD20 antibody molecule replaced by a different residue. The sites of greatest
       interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also
       contemplated. Conservative substitutions are shown in the Table below under the heading of "preferred
       substitutions". If such substitutions result in a change in biological activity, then more substantial changes,
       denominated "exemplary substitutions" in the Table, or as further described below in reference to amino acid
15   classes, may be introduced and the products screened.

TABLE of Amino Acid Substitutions

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the anti-CD20 antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human CD20. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the

5   recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

10   Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

       Nucleic acid molecules encoding amino acid sequence variants of the anti-CD20 antibody are

15   prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-CD20 antibody.

       It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so

20   as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased

25   complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., J. Exp Med. 176:1191-1195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al. Cancer Research 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement mediated lysis and

30   ADCC capabilities. See Stevenson et al. Anti-Cancer Drug Design 3:219-230 (1989).

       To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life

35   of the IgG molecule.

       Other antibody modifications

       Other modifications of the antibody are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol,

40   polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example,

5   liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences*, 16th edition, Oslo, A., Ed., (1980).

Screening for antibodies with the desired properties

Antibodies with certain biological characteristics may be selected as described in the Experimental
10   Examples.

The growth inhibitory effects of an anti-CD20 antibody of the invention may be assessed by methods known in the art, e.g., using cells which express CD20 either endogenously or following transfection with the CD20 gene. For example, tumor cell lines and CD20-transfected cells may treated with an anti-CD20 monoclonal antibody of the invention at various concentrations for a few days (e.g., 2-7) days
15   and stained with crystal violet or MTT or analyzed by some other colorimetric assay. Another method of measuring proliferation would be by comparing ^{3}H-thymidine uptake by the cells treated in the presence or absence an anti-CD20 antibody of the invention. After antibody treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriate positive controls include treatment of a selected cell line with a growth inhibitory antibody known to inhibit
20   growth of that cell line.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, *e.g.*, propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to control. A PI uptake assay can be performed in the absence of complement and immune effector cells. CD20-expressing tumor cells are incubated with medium alone or medium containing of the appropriate monoclonal antibody at e.g, about
25   10µg/ml . The cells are incubated for a 3 day time period. Following each treatment, cells are washed and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing
30   antibodies.

To screen for antibodies which bind to an epitope on CD20 bound by an antibody of interest, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody binds the same site or epitope as an anti-CD20 antibody of the invention. Alternatively, or
35   additionally, epitope mapping can be performed by methods known in the art . For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. The mutant antibody is initailly tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of CD20 can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

40

Vectors, Host Cells and Recombinant Methods

The invention also provides an isolated nucleic acid encoding a humanized CD20 binding antibody, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

The CD20 binding antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native CD20 binding antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the CD20 binding antibody.

(ii) Origin of replication

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus

5   survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic

       acid and hygromycin.

               Another example of suitable selectable markers for mammalian cells are those that enable the

       identification of cells competent to take up the CD20 binding antibody nucleic acid, such as DHFR,

       thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase,

10   ornithine decarboxylase, *etc.*

               For example, cells transformed with the DHFR selection gene are first identified by culturing all of

       the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR.

       An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line

       deficient in DHFR activity (e.g., ATCC CRL-9096).

15   Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed

       or co-transformed with DNA sequences encoding CD20 binding antibody, wild-type DHFR protein, and

       another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell

       growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic

       antibiotic, *e.g.,* kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

20   A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7

       (Stinchcomb *et al., Nature*, 282:39 (1979)). The *trp*1 gene provides a selection marker for a mutant strain of

       yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*,

       85:12 (1977). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective

       environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient

25   yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

               In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation

       of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf

       chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy

       expression vectors for secretion of mature recombinant human serum albumin by industrial strains of

30   *Kluyveromyces* have also been disclosed. Fleer *et al., Bio/Technology*, 9:968-975 (1991).

               (iv) Promoter component

               Expression and cloning vectors usually contain a promoter that is recognized by the host organism

       and is operably linked to the nucleic acid encoding the CD20 binding antibody. Promoters suitable for use

       with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline

35   phosphatase promoter, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter.

       However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will

       contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the CD20 binding antibody.

               Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich

       region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another

40   sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region

       where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may

       be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are

       suitably inserted into eukaryotic expression vectors.

5   Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-
phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate
dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase,
3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and
glucokinase.

10   Other yeast promoters, which are inducible promoters having the additional advantage of
transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2,
isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism,
metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and
galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP

15   73,657. Yeast enhancers also are advantageously used with yeast promoters.

CD20 binding antibody transcription from vectors in mammalian host cells is controlled, for
example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus,
adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a
retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian

20   promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided
such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction
fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human
cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA

25   in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446.
A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes et al., Nature
297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a
thymidine kinase promoter from herpes simplex virus. Alternatively, the Rous Sarcoma Virus long terminal
repeat can be used as the promoter.

30   (v) Enhancer element component

Transcription of a DNA encoding the CD20 binding antibody of this invention by higher eukaryotes
is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now
known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however,
one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side

35   of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer
on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18
(1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the
vector at a position 5' or 3' to the CD20 binding antibody-encoding sequence, but is preferably located at a
site 5' from the promoter.

40   (vi) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or
nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination
of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and,
occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain

5   nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA
       encoding CD20 binding antibody. One useful transcription termination component is the bovine growth
       hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

       (vii) Selection and transformation of host cells

       Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote,
10   yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria,
       such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*,
       e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.*, *Salmonella typhimurium*, *Serratia*,
       e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.*, *B.*
       licheniformis 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*,
15   and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains
       such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These
       examples are illustrative rather than limiting.

       Full length antibody, antibody fragments, and antibody fusion proteins can be produced in bacteria,
       in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic
20   antibody is conjugated to a cytotoxic agent (e.g., a toxin) and the immunoconjugate by itself shows
       effectiveness in tumor cell destruction. Full length antibodies have greater half life in circulation.
       Production in E. coli is faster and more cost efficient. For expression of antibody fragments and
       polypeptides in bacteria, see, e.g., U.S. 5,648,237 (Carter et. al.), U.S. 5,789,199 (Joly et al.), and U.S.
       5,840,523 (Simmons et al.) which describes translation initiation region (TIR) and signal sequences for
25   optimizing expression and secretion, these patents incorporated herein by reference. After expression, the
       antibody is isolated from the E. coli cell paste in a soluble fraction and can be purified through, e.g., a
       protein A or G column depending on the isotype. Final purification can be carried out similar to the process
       for purifying antibody expressed e.g., in CHO cells.

       In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable
30   cloning or expression hosts for CD20 binding antibody-encoding vectors. *Saccharomyces cerevisiae*, or
       common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However,
       a number of other genera, species, and strains are commonly available and useful herein, such as
       Schizosaccharomyces pombe; *Kluyveromyces* hosts such as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K.*
       bulgaricus (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum*
35   (ATCC 36,906), *K . thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP
       183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as
       Schwanniomyces occidentalis; and filamentous fungi such as, *e.g.*, *Neurospora*, *Penicillium*, *Tolypocladium*,
       and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

       Suitable host cells for the expression of glycosylated CD20 binding antibody are derived from
40   multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous
       baculoviral strains and variants and corresponding permissive insect host cells from hosts such as
       Spodoptera frugiperda (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila*
       melanogaster (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are
       publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori*

5   NPV, and such viruses may be used as the virus herein according to the present invention, particularly for
     transfection of *Spodoptera frugiperda* cells.

             Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized
     as hosts.

             However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture
10   (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey
     kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or
     293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.* 36:59 (1977)) ; baby
     hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.,
     Proc. Natl. Acad. Sci. USA* 77:4216 (1980)) ; mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251
15   (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC
     CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC
     CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75);
     human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells
     (Mather *et al., Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma
20   line (Hep G2).

             Host cells are transformed with the above-described expression or cloning vectors for CD20
     binding antibody production and cultured in conventional nutrient media modified as appropriate for
     inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

             (viii) Culturing the host cells
25   The host cells used to produce the CD20 binding antibody of this invention may be cultured in a
     variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium
     ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are
     suitable for culturing the host cells. In addition, any of the media described in Ham *et al., Meth. Enz.* 58:44
     (1979), Barnes *et al., Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762;
30   4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture
     media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other
     growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride,
     calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and
     thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds
35   usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source.
     Any other necessary supplements may also be included at appropriate concentrations that would be known to
     those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously
     used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

             (ix) Purification of antibody
40   When using recombinant techniques, the antibody can be produced intracellularly, in the
     periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first
     step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation
     or ultrafiltration. Carter *et al., Bio/Technology* 10:163-167 (1992) describe a procedure for isolating
     antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the

5   presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min.
       Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants
       from such expression systems are generally first concentrated using a commercially available protein
       concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor
       such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be
10   included to prevent the growth of adventitious contaminants.

               The antibody composition prepared from the cells can be purified using, for example,
       hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity
       chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand
       depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody.
15   Protein A can be used to purify antibodies that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et*
       al., J. Immunol. Meth. 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$
       (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often
       agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or
       poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved
20   with agarose. Where the antibody comprises a $C_H 3$ domain, the Bakerbond ABX™resin (J. T. Baker,
       Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on
       an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica,
       chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as
       a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also
25   available depending on the antibody to be recovered.

               Following any preliminary purification step(s), the mixture comprising the antibody of interest and
       contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer
       at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.,* from about 0-0.25M
       salt).

30

Antibody conjugates

               The antibody may be conjugated to a cytotoxic agent such as a toxin or a radioactive isotope. In
       certain embodiments, the toxin is calicheamicin, a maytansinoid, a dolastatin, auristatin E and analogs or
       derivatives thereof, are preferable.
35   Preferred drugs/toxins include DNA damaging agents, inhibitors of microtubule polymerization or
       depolymerization and antimetabolites. Preferred classes of cytotoxic agents include, for example, the
       enzyme inhibitors such as dihydrofolate reductase inhibitors, and thymidylate synthase inhibitors, DNA
       intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the
       mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, the
40   podophyllotoxins and differentiation inducers. Particularly useful members of those classes include, for
       example, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine
       arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, doxorubicin, N-(5,5-
       diacetoxypentyl)doxorubicin, morpholino-doxorubicin, 1-(2-choroehthyl)-1,2-dimethanesulfonyl hydrazide,
       N^8-acetyl spermidine, aminopterin methopterin, esperamicin, mitomycin C, mitomycin A, actinomycin,

5   bleomycin, carminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives
    such as etoposide or etoposide phosphate, vinblastine, vincristine, vindesine, taxol, taxotere, retinoic acid,
    butyric acid, N^8-acetyl spermidine, camptothecin, calicheamicin, bryostatins, cephalostatins, ansamitocin,
    actosin, maytansinoids such as DM-1, maytansine, maytansinol, N-desmethyl-4,5-desepoxymaytansinol, C-
    19-dechloromaytansinol, C-20-hydroxymaytansinol, C-20-demethoxymaytansinol, C-9-SH maytansinol, C-
10 14-alkoxymethylmaytansinol, C-14-hydroxy or acetyloxymethlmaytansinol, C-15-
    hydroxy/acetyloxymaytansinol, C-15-methoxymaytansinol, C-18-N-demethylmaytansinol and 4,5-
    deoxymaytansinol, auristatins such as auristatin E, M, PHE and PE; dolostatins such as dolostatin A,
    dolostatin B, dolostatin C, dolostatin D, dolostatin E (20-epi and 11-epi), dolostatin G, dolostatin H,
    dolostatin I, dolostatin 1, dolostatin 2, dolostatin 3, dolostatin 4, dolostatin 5, dolostatin 6, dolostatin 7,
15 dolostatin 8, dolostatin 9, dolostatin 10, deo-dolostatin 10, dolostatin 11, dolostatin 12, dolostatin 13,
    dolostatin 14, dolostatin 15, dolostatin 16, dolostatin 17, and dolostatin 18; cephalostatins such as
    cephalostatin 1, cephalostatin 2, cephalostatin 3, cephalostatin 4, cephalostatin 5, cephalostatin 6,
    cephalostatin 7, 25'-epi-cephalostatin 7, 20-epi-cephalostatin 7, cephalostatin 8, cephalostatin 9,
    cephalostatin 10, cephalostatin 11,cephalostatin 12,cephalostatin 13,cephalostatin 14, cephalostatin
20 15,cephalostatin 16,cephalostatin 17, cephalostatin 18, and cephalostatin 19..

    Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine
    was first isolated from the east African shrub *Maytenus serrata* (U.S. Patent No. 3,896,111). Subsequently,
    it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3
    maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues
25 thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608;
    4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821;
    4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533, the
    disclosures of which are hereby expressly incorporated by reference.

    Maytansine and maytansinoids have been conjugated to antibodies specifically binding to tumor
30 cell antigens. Immunoconjugates containing maytansinoids and their therapeutic use are disclosed, for
    example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of
    which are hereby expressly incorporated by reference. Liu *et al.*, Proc. Natl. Acad. Sci. USA 93:8618-8623
    (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal
    antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic
35 towards cultured colon cancer cells, and showed antitumor activity in an *in vivo* tumor growth assay. Chari
    et al. Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was
    conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell
    lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/*neu* oncogene.

    There are many linking groups known in the art for making antibody-maytansinoid conjugates,
40 including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, and Chari
    et al. Cancer Research 52: 127-131 (1992). The linking groups include disufide groups, thioether groups,
    acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the
    above-identified patents, disulfide and thioether groups being preferred.

5   Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium

10   derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson *et al.*, *Biochem. J.* 173:723-737 [1978]) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

15   The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hyrdoxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position

20   of maytansinol or a maytansinol analogue.

          Calicheamicin

          Another immunoconjugate of interest comprises an CD20 binding antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-

25   stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman *et al. Cancer Research* 53: 3336-3342 (1993), Lode *et al. Cancer Research* 58: 2925-2928 (1998) and the aforementioned

30   U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

35   *Radioactive isotopes*

          For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated anti-CD20 antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for diagnosis, it may comprise a radioactive atom for scintigraphic studies, for

40   example tc^{99m} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

          The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino

5   acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as tc^{99m} or I^{123}, $.Re^{186}$, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57 can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

10   Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium

15   derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a

20   "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992); U.S. Patent No. 5,208,020) may be used.

Therapeutic Uses of the CD20 binding Antibodies

25   The CD20 binding antibodies of the invention are useful to treat a number of malignant and non-malignant diseases including autoimmune diseases and related conditions, and CD20 positive cancers including B cell lymphomas and leukemias. Stem cells (B-cell progenitors) in bone marrow lack the CD20 antigen, allowing healthy B-cells to regenerate after treatment and return to normal levels within several months.

30   Autoimmune diseases or autoimmune related conditions include arthritis (rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), psoriasis, dermatitis including atopic dermatitis; chronic autoimmune urticaria, polymyositis/dermatomyositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (IBD) (Crohn's disease, ulcerative colitis), respiratory distress syndrome, adult respiratory distress syndrome (ARDS),

35   meningitis, allergic rhinitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE), lupus (including nephritis, non-renal, discoid, alopecia), juvenile onset diabetes, multiple sclerosis, allergic encephalomyelitis, immune responses associated with acute and delayed hypersensitivity mediated by

40   cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis, agranulocytosis, vasculitis (including ANCA), aplastic anemia, Coombs positive anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, CNS inflammatory

5   disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet disease, Castleman's syndrome, Goodpasture's Syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection (including pretreatment for high panel reactive antibody titers, IgA deposit in tissues, etc), graft versus host disease

10   (GVHD), pemphigoid bullous, pemphigus (all including vulgaris, foliatis), autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune

15   endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM) and Sheehan's syndrome; autoimmune hepatitis, Lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barre' Syndrome, Large

20   Vessel Vasculitis (including Polymyalgia Rheumatica and Giant Cell (Takayasu's) Arteritis), Medium Vessel Vasculitis (including Kawasaki's Disease and Polyarteritis Nodosa), ankylosing spondylitis, Berger's Disease (IgA nephropathy), Rapidly Progressive Glomerulonephritis, Primary biliary cirrhosis, Celiac sprue (gluten enteropathy), Cryoglobulinemia, ALS, coronary artery disease.

         CD20 positive cancers are those comprising abnormal proliferation of cells that express CD20 on

25   the cell surface. The CD20 positive B cell neoplasms include CD20-positive Hodgkin's disease including lymphocyte predominant Hodgkin's disease (LPHD); non-Hodgkin's lymphoma (NHL); follicular center cell (FCC) lymphomas; acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); Hairy cell leukemia. The non-Hodgkins lymphoma include low grade/follicular non-Hodgkin's lymphoma (NHL), small lymphocytic lymphoma (SLL), intermediate grade/follicular NHL, intermediate grade diffuse NHL,

30   high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, plasmacytoid lymphocytic lymphoma, mantle cell lymphoma, AIDS- related lymphoma and Waldenstrom's macroglobulinemia. Treatment of relapses of these cancers are also contemplated. LPHD is a type of Hodgkin's disease that tends to relapse frequently despite radiation or chemotherapy treatment and is characterized by CD20-positive malignant cells. CLL is one of four major types of

35   leukemia. A cancer of mature B-cells called lymphocytes, CLL is manifested by progressive accumulation of cells in blood, bone marrow and lymphatic tissues.

         In specific embodiments, the humanized CD20 binding antibodies and functional fragments thereof are used to treat non-Hodgkin's lymphoma (NHL), lymphocyte predominant Hodgkin's disease (LPHD), small lymphocytic lymphoma (SLL), chronic lymphocytic leukemia, rheumatoid arthritis and juvenile

40   rheumatoid arthritis, systemic lupus erythematosus (SLE) including lupus nephritis, Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome, Sjorgen's syndrome and glomerulonephritis.

5   The humanized CD20 binding antibodies or functional fragments thereof are useful as a single-

agent treatment in, e.g., for relapsed or refractory low-grade or follicular, CD20-positive, B-cell NHL, or can

be administered to patients in conjunction with other drugs in a multi drug regimen.

The parameters for assessing efficacy or success of treatment of the neoplasm will be known to the

between six and 10 years following numerous periods of remission and relapse. In one embodiment, the

10   humanized CD20 binding antibodies or functional fragments thereof are used to treat indolent NHL.

The parameters for assessing efficacy or success of treatment of the neoplasm will be known to the

physician of skill in the appropriate disease. Generally, the physician of skill will look for reduction in the

signs and symptoms of the specific disease. Parameters can include median time to disease progression,

time in remission, stable disease.

15   The following references describe lymphomas and CLL, their diagnoses, treatment and standard

medical procedures for measuring treatment efficacy.

The following references describe lymphomas and CLL, their diagnoses, treatment and standard

medical procedures for measuring treatment efficacy. Canellos GP, Lister, TA, Sklar JL: *The Lymphomas.*

W.B.Saunders Company, Philadelphia, 1998; van Besien K and Cabanillas, F: Clinical Manifestations,

20   Staging and Treatment of Non-Hodgkin's Lymphoma, Chap. 70, pp 1293-1338, in: *Hematology , Basic*

Principles and Practice, 3rd ed. Hoffman et al. (editors). Churchill Livingstone, Philadelphia, 2000; and Rai,

K and Patel, D:Chronic Lymphocytic Leukemia, Chap. 72, pp 1350-1362, in: *Hematology , Basic Principles*

and Practice, 3rd ed. Hoffman et al. (editors). Churchill Livingstone, Philadelphia, 2000.

The parameters for assessing efficacy or success of treatment of an autoimmune or autoimmune

25   related disease will be known to the physician of skill in the appropriate disease. Generally, the physician of

skill will look for reduction in the signs and symptoms of the specific disease. The following are by way of

examples.

In one embodiment, the antibodies of the invention are useful to treat rheumatoid arthritis. RA is

characterized by inflammation of multiple joints, cartilage loss and bone erosion that leads to joint

30   destruction and ultimately reduced joint function. Additionally, since RA is a systemic disease, it can have

effects in other tissues such as the lungs, eyes and bone marrow. Fewer than 50 percent of patients who

have had RA for more than 10 years can continue to work or function normally on a day-to-day basis.

The antibodies can be used as first-line therapy in patients with early RA (i.e., methotrexate (MTX)

naive) and as monotherapy, or in combination with, e.g., MTX or cyclophosphamide. Or, the antibodies can

35   be used in treatment as second-line therapy for patients who were DMARD and/or MTX refractory, and as

monotherapy or in combination with, e.g., MTX. The humanized CD20 binding antibodies are useful to

prevent and control joint damage, delay structural damage, decrease pain associated with inflammation in

RA, and generally reduce the signs and symptoms in moderate to severe RA. The RA patient can be treated

with the humanized CD20 antibody prior to, after or together with treatment with other drugs used in

40   treating RA (see combination therapy below). In one embodiment, patients who had previously failed

disease-modifying antirheumatic drugs and/or had an inadequate response to methotrexate alone are treated

with a humanized CD20 binding antibody of the invention. In one embodiment of this treatment, the patients

are in a 17-day treatment regimen receiving humanized CD20 binding antibody alone (1g iv infusions on

5   days 1 and 15); CD20 binding antibody plus cyclophosphamide (750mg iv infusion days 3 and 17); or CD20

    binding antibody plus methotrexate.

            One method of evaluating treatment efficacy in RA is based on American College of Rheumatology

    (ACR) criteria, which measures the percentage of improvement in tender and swollen joints, among other

    things. The RA patient can be scored at for example, ACR 20 (20 percent improvement) compared with no

10 antibody treatment (e.g,, baseline before treatment) or treatment with placebo. Other ways of evaluating the

    efficacy of antibody treatment include X-ray scoring such as the Sharp X-ray score used to score structural

    damage such as bone erosion and joint space narrowing. Patients can also be evaluated for the prevention of

    or improvement in disability based on Health Assessment Questionnaire [HAQ] score, AIMS score, SF-36 at

    time periods during or after treatment. The ACR 20 criteria may include 20% improvement in both tender

15 (painful) joint count and swollen joint count plus a 20% improvement in at least 3 of 5 additional measures:

        1.   patient's pain assessment by visual analog scale (VAS),
        2.   patient's global assessment of disease activity (VAS),
        3.   physician's global assessment of disease activity (VAS),
        4.   patient's self-assessed disability measured by the Health Assessment Questionnaire,
20   and
        5.   acute phase reactants, CRP or ESR.

    The ACR 50 and 70 are defined analogously. Preferably, the patient is administered an amount of a CD20

    binding antibody of the invention effective to achieve at least a score of ACR 20, preferably at least ACR 30,

25 more preferably at least ACR50, even more preferably at least ACR70, most preferably at least ACR 75 and

    higher.

            Psoriatic arthritis has unique and distinct radiographic features. For psoriatic arthritis, joint erosion

    and joint space narrowing can be evaluated by the Sharp score as well. The humanized CD20 binding

    antibodies of the invention can be used to prevent the joint damage as well as reduce disease signs and

30 symptoms of the disorder.

            Yet another aspect of the invention is a method of treating Lupus or SLE by administering to the

    patient suffering from SLE, a therapeutically effective amount of a humanized CD20 binding antibody of the

    invention. SLEDAI scores provide a numerical quantitation of disease activity. The SLEDAI is a weighted

    index of 24 clinical and laboratory parameters known to correlate with disease activity, with a numerical

35 range of 0-103. see Bryan Gescuk & John Davis, "Novel therapeutic agent for systemic lupus

    erythematosus" in Current Opinion in Rheumatology 2002, 14:515-521. Antibodies to double-stranded DNA

    are believed to cause renal flares and other manifestations of lupus. Patients undergoing antibody treatment

    can be monitored for time to renal flare, which is defined as a significant, reproducible increase in serum

    creatinine, urine protein or blood in the urine. Alternatively or in addition, patients can be monitored for

40 levels of antinuclear antibodies and antibodies to double-stranded DNA. Treatments for SLE include high-

    dose corticosteroids and/or cyclophosphamide (HDCC).

            Spondyloarthropathies are a group of disorders of the joints, including ankylosing spondylitis,

    psoriatic arthritis and Crohn's disease. Treatment success can be determined by validated patient and

    physician global assessment measuring tools.

45   Various medications are used to treat psoriasis; treatment differs directly in relation to disease

    severity. Patients with a more mild form of psoriasis typically utilize topical treatments, such as topical

5   steroids, anthralin, calcipotriene, clobetasol, and tazarotene, to manage the disease while patients with
     moderate and severe psoriasis are more likely to employ systemic (methotrexate, retinoids, cyclosporine,
     PUVA and UVB) therapies. Tars are also used. These therapies have a combination of safety concerns, time
     consuming regimens, or inconvenient processes of treatment. Furthermore, some require expensive
     equipment and dedicated space in the office setting. Systemic medications can produce serious side effects,
10   including hypertension, hyperlipidemia, bone marrow suppression, liver disease, kidney disease and
     gastrointestinal upset. Also, the use of phototherapy can increase the incidence of skin cancers. In addition
     to the inconvenience and discomfort associated with the use of topical therapies, phototherapy and systemic
     treatments require cycling patients on and off therapy and monitoring lifetime exposure due to their side
     effects.

15   Treatment efficacy for psoriasis is assessed by monitoring changes in clinical signs and symptoms
     of the disease including Physician's Global Assessment (PGA) changes and Psoriasis Area and Severity
     Index (PASI) scores, Psoriasis Symptom Assessment (PSA), compared with the baseline condition. The
     patient can be measured periodically throughout treatment on the Visual analog scale used to indicate the
     degree of itching experienced at specific time points.

20   Patients may experience an infusion reaction or infusion-related symptoms with their first infusion
     of a therapeutic antibody. These symptoms vary in severity and generally are reversible with medical
     intervention. These symptoms include but are not limited to, flu-like fever, chills/rigors, nausea, urticaria,
     headache, bronchospasm, angioedema. It would be desirable for the disease treatment methods of the
     present invention to minimize infusion reactions. Thus, another aspect of the invention is a method of
25   treating the diseases disclosed by administering a humanized CD20 binding antibody wherein the antibody
     has reduced or no complement dependent cytotoxicity and results in reduced infusion related symptoms as
     compared to treatment with Rituxan®. In one embodiment, the humanized CD20 binding antibody is
     2H7.v116.

30   *Dosage*
             Depending on the indication to be treated and factors relevant to the dosing that a physician of skill
     in the field would be familiar with, the antibodies of the invention will be administered at a dosage that is
     efficacious for the treatment of that indication while minimizing toxicity and side effects. For the treatment
     of a CD20 positive cancer or an autoimmune disease, the therapeutically effective dosage will be in the
35   range of about 250mg/m^2 to about 400 mg/m^2 or 500 mg/m^2, preferably about 250-375mg/m^2. In one
     embodiment, the dosage range is 275-375 mg/m^2. In one embodiment of the treatment of a CD20 positive B
     cell neoplasm, the antibody is administered at a range of 300-375 mg/m^2. For the treatment of patients
     suffering from B-cell lymphoma such as non-Hodgkins lymphoma, in a specific embodiment, the anti-CD20
     antibodies and humanized anti-CD20 antibodies of the invention will be administered to a human patient at a
40   dosage of 10mg/kg or 375mg/m^2. For treating NHL, one dosing regimen would be to administer one dose of
     the antibody composition a dosage of 10mg/kg in the first week of treatment, followed by a 2 week interval,
     then a second dose of the same amount of antibody is administered. Generally, NHL patients receive such
     treatment once during a year but upon recurrence of the lymphoma, such treatment can be repeated. In
     another dosing regimen, patients treated with low-grade NHL receive four weeks of a version of humanized

5   2H7, preferably v16 (375 mg/m2 weekly) followed at week five by three additional courses of the antibody
plus standard CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) or CVP
(cyclophosphamide, vincristine, prednisone) chemotherapy, which was given every three weeks for three
cycles.

For treating rheumatoid arthritis, in one embodiment, the dosage range for the humanized antibody
10   is 125mg/m^2 (equivalent to about 200mg/dose) to 600mg/m^2, given in two doses, e.g., the first dose of
200mg is administered on day one followed by a second dose of 200mg on day 15. In different
embodiments, the dosage is 250mg/dose, 275mg, 300mg, 325mg, 350mg, 375mg, 400mg, 425mg, 450mg,
475mg, 500mg, 525mg, 550mg, 575mg, 600mg.

In treating disease, the CD20 binding antibodies of the invention can be administered to the patient
15   chronically or intermittently, as determined by the physician of skill in the disease.

A patient administered a drug by intravenous infusion or subcutaneously may experience adverse
events such as fever, chills, burning sensation, asthenia and headache. To alleviate or minimize such
adverse events, the patient may receive an initial conditioning dose(s) of the antibody followed by a
therapeutic dose. The conditioning dose(s) will be lower than the therapeutic dose to condition the patient to
20   tolerate higher dosages.

Route of administration

The CD20 binding antibodies are administered to a human patient in accord with known methods,
such as by intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by
25   subcutaneous, intramuscular, intraperitoneal, intracerobrospinal, intra-articular, intrasynovial, intrathecal, or
inhalation routes, generally by intravenous or subcutaneous administration.

In on embodiment, the humanized 2H7 antibody is administered by intravenous infusion with 0.9%
sodium chloride solution as an infusion vehicle.

30   *Combination Therapy*

In treating the B cell neoplasms described above, the patient can be treated with the CD20 binding
antibodies of the present invention in conjunction with one or more therapeutic agents such as a
chemotherapeutic agent in a multidrug regimen. The CD20 binding antibody can be administered
concurrently, sequentially, or alternating with the chemotherapeutic agent, or after non-responsiveness with
35   other therapy. Standard chemotherapy for lymphoma treatment may include cyclophosphamide, cytarabine,
melphalan and mitoxantrone plus melphalan. CHOP is one of the most common chemotherapy regimens
for treating Non-Hodgkin's lymphoma. The following are the drugs used in the CHOP regimen:
cyclophosphamide (brand names cytoxan, neosar); adriamycin (doxorubicin / hydroxydoxorubicin);
vincristine (Oncovin); and prednisolone (sometimes called Deltasone or Orasone). In particular
40   embodiments, the CD20 binding antibody is administered to a patient in need thereof in combination with
one or more of the following chemotherapeutic agents of doxorubicin, cyclophosphamide, vincristine and
prednisolone. In a specific embodiment, a patient suffering from a lymphoma (such as a non-Hodgkin's
lymphoma) is treated with an anti-CD20 antibody of the present invention in conjunction with CHOP
(cyclophosphamide, doxorubicin, vincristine and prednisone) therapy. In another embodiment, the cancer

5   patient can be treated with a humanized CD20 binding antibody of the invention in combination with CVP
       (cyclophosphamide, vincristine, and prednisone) chemotherapy. In a specific embodiment, the patient
       suffering from CD20-positive NHL is treated with humanized 2H7.v16 in conjunction with CVP. In a
       specific embodiment of the treatment of CLL, the CD20 binding antibody is administered in conjunction
       with chemotherapy with one or both of fludarabine and cytoxan.

10   In treating the autoimmune diseases or autoimmune related conditions described above, the patient
       can be treated with the CD20 binding antibodies of the present invention in conjunction with a second
       therapeutic agent, such as an immunosuppressive agent, such as in a multi drug regimen. The CD20 binding
       antibody can be administered concurrently, sequentially or alternating with the immunosuppressive agent or
       upon non-responsiveness with other therapy. The immunosuppressive agent can be administered at the

15   same or lesser dosages than as set forth in the art. The preferred adjunct immunosuppressive agent will
       depend on many factors, including the type of disorder being treated as well as the patient's history.

               "Immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to
       suppress or mask the immune system of a patient. Such agents would include substances that suppress
       cytokine production, down regulate or suppress self-antigen expression, or mask the MHC antigens.

20   Examples of such agents include steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone,
       and dexamethasone; 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077), azathioprine
       (or cyclophosphamide, if there is an adverse reaction to azathioprine); bromocryptine; glutaraldehyde (which
       masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC
       antigens and MHC fragments; cyclosporin A; cytokine or cytokine receptor antagonists including anti-

25   interferon-γ, -β, or -α antibodies; anti-tumor necrosis factor-α antibodies; anti-tumor necrosis factor-β
       antibodies; anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-L3T4 antibodies;
       heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a
       antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90);
       streptokinase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin;

30   rapamycin; T-cell receptor (U.S. Pat. No. 5,114,721); T-cell receptor fragments (Offner *et al., Science*
       251:430-432 (1991); WO 90/11294; and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as
       T10B9.

               For the treatment of rheumatoid arthritis, the patient can be treated with a CD20 antibody of the
       invention in conjunction with any one or more of the following drugs: DMARDS (disease-modifying anti-

35   rheumatic drugs (e.g., methotrexate), NSAI or NSAID (non-steroidal anti-inflammatory drugs),
       HUMIRA™ (adalimumab; Abbott Laboratories), ARAVA® (leflunomide), REMICADE® (infliximab;
       Centocor Inc., of Malvern, Pa), ENBREL (etanercept; Immunex, WA), COX-2 inhibitors. DMARDs
       commonly used in RA are hydroxycloroquine, sulfasalazine, methotrexate, leflunomide, etanercept,
       infliximab, azathioprine, D-penicillamine, Gold (oral), Gold (intramuscular), minocycline, cyclosporine,

40   Staphylococcal protein A immunoadsorption. Adalimumab is a human monoclonal antibody that binds to
       TNFα. Infliximab is a chimeric monoclonal antibody that binds to TNFα. Etanercept is an
       "immunoadhesin" fusion protein consisting of the extracellular ligand binding portion of the human 75 kD
       (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of a human IgG1. For conventional
       treatment of RA, see, e.g., "Guidelines for the management of rheumatoid arthritis" *Arthritis & Rheumatism*

5   46(2): 328-346 (February, 2002). In a specific embodiment, the RA patient is treated with a CD20 antibody
       of the invention in conjunction with methotrexate (MTX). An exemplary dosage of MTX is about 7.5–
       25 mg/kg/wk. MTX can be administered orally and subcutaneously.

              For the treatment of ankylosing spondylitis, psoriatic arthritis and Crohn's disease, the patient can
       be treated with a CD20 binding antibody of the invention in conjunction with, for example, Remicade®
10   (infliximab; from Centocor Inc., of Malvern, Pa.), ENBREL (etanercept; Immunex, WA).

              Treatments for SLE include high-dose corticosteroids and/or cyclophosphamide (HDCC).

              For the treatment of psoriasis, patients can be administered a CD20 binding antibody in conjunction
       with topical treatments, such as topical steroids, anthralin, calcipotriene, clobetasol, and tazarotene, or with
       methotrexate, retinoids, cyclosporine, PUVA and UVB therapies. In one embodiment, the psoriasis patient
15   is treated with the CD20 binding antibody sequentially or concurrently with cyclosporine.

Pharmaceutical Formulations

              Therapeutic formulations of the CD20-binding antibodies used in accordance with the present
       invention are prepared for storage by mixing an antibody having the desired degree of purity with optional
20   pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th
       edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable
       carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and
       include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and
       methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride;
25   benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as
       methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular
       weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or
       immunoglobulins; hydrophilic polymers such as olyvinylpyrrolidone; amino acids such as glycine,
       glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other
30   carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as
       sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-
       protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol
       (PEG).

              Exemplary anti-CD20 antibody formulations are described in WO98/56418, expressly incorporated
35   herein by reference. Another formulation is a liquid multidose formulation comprising the anti-CD20
       antibody at 40 mg/mL, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH
       5.0 that has a minimum shelf life of two years storage at 2-8°C. Another anti-CD20 formulation of interest
       comprises 10mg/mL antibody in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate,
       0.7mg/mL polysorbate 80, and Sterile Water for Injection, pH 6.5. Yet another aqueous pharmaceutical
40   formulation comprises 10-30 mM sodium acetate from about pH 4.8 to about pH 5.5, preferably at pH5.5,
       polysorbate as a surfactant in a an amount of about 0.01-0.1% v/v, trehalose at an amount of about 2-10%
       w/v, and benzyl alcohol as a preservative (U.S. 6,171,586). Lyophilized formulations adapted for
       subcutaneous administration are described in WO97/04801. Such lyophilized formulations may be

5   reconstituted with a suitable diluent to a high protein concentration and the reconstituted formulation may be

         administered subcutaneously to the mammal to be treated herein.

                 One formulation for the humanized 2H7 variants is antibody at 12-14 mg/mL in 10 mM histidine,

         6% sucrose, 0.02% polysorbate 20, pH 5.8.

                 In a specific embodiment, 2H7 variants and in particular 2H7.v16 is formulated at 20mg/mL

10   antibody in 10mM histidine sulfate, 60mg/ml sucrose., 0.2 mg/ml polysorbate 20, and Sterile Water for

         Injection, at pH5.8.   (

                 The formulation herein may also contain more than one active compound as necessary for the

         particular indication being treated, preferably those with complementary activities that do not adversely

         affect each other. For example, it may be desirable to further provide a cytotoxic agent, chemotherapeutic

15   agent, cytokine or immunosuppressive agent (e.g. one which acts on T cells, such as cyclosporin or an

         antibody that binds T cells, e.g. one which binds LFA-1). The effective amount of such other agents

         depends on the amount of antibody present in the formulation, the type of disease or disorder or treatment,

         and other factors discussed above. These are generally used in the same dosages and with administration

         routes as described herein or about from 1 to 99% of the heretofore employed dosages.

20   The active ingredients may also be entrapped in microcapsules prepared, for example, by

         coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-

         microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems

         (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in

         macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol,

25   A. Ed. (1980).

                 Sustained-release preparations may be prepared. Suitable examples of sustained-release

         preparations include semi-permeable matrices of solid hydrophobic polymers containing the antagonist,

         which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-

         release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or

30   poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-

         glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as

         the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and

         leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

                 The formulations to be used for in vivo administration must be sterile. This is readily accomplished

35   by filtration through sterile filtration membranes.

Articles of Manufacture and Kits

                 Another embodiment of the invention is an article of manufacture containing materials useful for

         the treatment of autoimmune diseases and related conditions and CD20 positive cancers such as non-

40   Hodgkin's lymphoma. The article of manufacture comprises a container and a label or package insert on or

         associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The

         containers may be formed from a variety of materials such as glass or plastic. The container holds a

         composition which is effective for treating the condition and may have a sterile access port (for example the

         container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection

5   needle). At least one active agent in the composition is a CD20 binding antibody of the invention. The label
    or package insert indicates that the composition is used for treating the particular condition. The label or
    package insert will further comprise instructions for administering the antibody composition to the patient.
    Package insert refers to instructions customarily included in commercial packages of therapeutic products,
    that contain information about the indications, usage, dosage, administration, contraindications and/or
10 warnings concerning the use of such therapeutic products. In one embodiment, the package insert indicates
    that the composition is used for treating non-Hodgkins' lymphoma.

           Additionally, the article of manufacture may further comprise a second container comprising a
    pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered
    saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a
15 commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

           Kits are also provided that are useful for various purposes , e.g., for B-cell killing assays, as a
    positive control for apoptosis assays, for purification or immunoprecipitation of CD20 from cells. For
    isolation and purification of CD20, the kit can contain an anti-CD20 antibody coupled to beads (e.g.,
    sepharose beads). Kits can be provided which contain the antibodies for detection and quantitation of CD20
20 in vitro, e.g. in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a
    container and a label or package insert on or associated with the container. The container holds a
    composition comprising at least one anti-CD20 antibody of the invention. Additional containers may be
    included that contain, e.g., diluents and buffers, control antibodies. The label or package insert may provide
    a description of the composition as well as instructions for the intended in vitro or diagnostic use.
25

Cynomolgus monkey CD20

           The invention also provides an isolated nucleic acid comprising the nucleotide sequence of SEQ ID
    NO.: _ of the Cynomolgus monkey CD20 as shown in FIG. 19. In one embodiment, the nucleic acid is a
    cDNA. In one embodiment, the nucleic acid encoding the monkey CD20 is in an expression vector for
30 expression in a host cell. The nucleotide sequence of SEQ ID NO.: _ in the expression vector is operably
    linked to an expression control sequence such as a promoter or promoter and enhancer. The expression
    control sequence can be can be the native sequence normally associated with the Cynomolgus CD20 gene, or
    heterologous to the gene. Also provided is an isolated polypeptide comprising the amino acid sequence
    [SEQ ID NO. _ ; FIG. 19 & 20] of the Cynomolgus monkey CD20, as well as host cells containing the
35 Cynomolgus CD20 nucleic acid. In one aspect the host cells are eukaryotic cells, e.g., CHO cells. Fusion
    proteins comprising the Cynomolgus CD20 amino acid sequence or fragments of the sequence are also
    contemplated.

Experimental Examples

40   Example 1

           Humanization of 2H7 anti-CD20 murine monoclonal antibody
           Humanization of the murine anti-human CD20 antibody, 2H7 (also referred to herein as m2H7, m
    for murine), was carried out in a series of site-directed mutagenesis steps. The murine 2H7 antibody
45 variable region sequences and the chimeric 2H7 with the mouse V and human C have been described, see,

                                              45

e.g., U.S. patents 5,846,818 and 6,204,023. The CDR residues of 2H7 were identified by comparing the amino acid sequence of the murine 2H7 variable domains (disclosed in U.S. 5,846,818) with the sequences of known antibodies (Kabat et al., Sequences of proteins of immunological interest, Ed. 5. Public Health Service, National Institutes of Health, Bethesda, MD (1991)). The CDR regions for the light and heavy chains were defined based on sequence hypervariability (Kabat et al., *supra*) and are shown in Fig. 1A and Fig. 1B, respectively. Using synthetic oligonucleotides (Table 1), site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci.* 82:488-492 (1985)) was used to introduce all six of the murine 2H7 CDR regions into a complete human Fab framework corresponding to a consensus sequence $V_\kappa I, V_H III$ (V_L kappa subgroup I, V_H subgroup III) contained on plasmid pVX4 (Fig. 2).

The phagemid pVX4 (Fig. 2) was used for mutagenesis as well as for expression of F(ab)s in E. coli. Based on the phagemid pb0720, a derivative of pB0475 (Cunningham et al., Science 243: 1330-1336 (1989)), pVX4 contains a DNA fragment encoding a humanized consensus κ-subgroup I light chain ($V_L \kappa I$-C_L) and a humanized consensus subgroup III heavy chain ($V_H III$-$C_H 1$) anti-IFN-α (interferon α) antibody. pVX4 also has an alkaline phosphatase promotor and Shine-Dalgarno sequence both derived from another previously described pUC119-based plasmid, pAK2 (Carter et al., Proc. Natl. Acad. Sci. USA 89: 4285 (1992)). A unique SpeI restriction site was introduced between the DNA encoding for the F(ab) light and heavy chains. The first 23 amino acids in both anti-IFN-α heavy and light chains are the StII secretion signal sequence (Chang et al., *Gene* 55: 189-196 (1987)).

To construct the CDR-swap version of 2H7 (2H7.v2), site-directed mutagenesis was performed on a deoxyuridine-containing template of pVX4; all six CDRs of anti-IFN-α were changed to the murine 2H7 CDRs. The resulting molecule is referred to as humanized 2H7 version 2 (2H7.v2), or the "CDR-swap version" of 2H7; it has the m2H7 CDR residues with the consensus human FR residues shown in Figures 1A and 1B. Humanized 2H7.v2 was used for further humanization.

Table 1 shows the oligonucleotide sequence used to create each of the murine 2H7 (m2H7) CDRs in the H and L chain. For example, the CDR-H1 oligonucleotide was used to recreate the m2H7 H chain CDR1. CDR-H1, CDR-H2 and CDR-H3 refers to the H chain CDR1, CDR2 and CDR3, respectively; similarly, CDR-L1, CDR-L2 and CDR-L3 refers to each of the L chain CDRs. The substitutions in CDR-H2 were done in two steps with two oligonucleotides, CDR-H2A and CDR-H2B.

Table 1. Oligonucleotide sequences used for construction of the CDR-swap of murine 2H7 CDRs into a human framework in pVX4. Residues changed by each oligonucleotide are underlined.

Substitution	Oligonucleotide sequence
CDR-H1	C TAC ACC TTC ACG <u>AGC</u> TAT <u>AAC</u> <u>ATG</u> CAC TGG GTC CG (SEQ ID NO.)
CDR-H2A	G ATT AAT CCT GAC <u>AAC</u> <u>GGC</u> <u>GAC</u> ACG <u>AGC</u> TAT AAC CAG <u>AAG</u> TTC AAG GGC CG (SEQ ID NO. _)
CDR-H2B	GAA TGG GTT GCA <u>GCG</u> ATC <u>TAT</u> CCT <u>GGC</u> AAC GGC GAC AC (SEQ ID NO. _)
CDR-H3	AT TAT TGT GCT CGA GTG <u>GTC</u> <u>TAC</u> <u>TAT</u> AGC AAC AGC <u>TAC</u> <u>TGG</u> TAC TTC GAC <u>GTC</u> TGG GGT CAA GGA (SEQ ID NO. _)
CDR-L1	C TGC ACA GCC AGC <u>TCT</u> TCT <u>GTC</u> AGC TAT ATG CAT TG (SEQ ID NO. _)
CDR-L2	AA CTA CTG ATT TAC <u>GCT</u> <u>CCA</u> <u>TCG</u> AAC CTC <u>GCG</u> TCT GGA GTC C (SEQ ID NO. _)
CDR-L3	TAT TAC TGT CAA CAG <u>TGG</u> <u>AGC</u> <u>TTC</u> <u>AAT</u> CCG <u>CCC</u> ACA TTT GGA

CAG (SEQ ID NO. _)

For comparison with humanized constructs, a plasmid expressing a chimeric 2H7 Fab (containing murine V_L and V_H domains, and human C_L and CH_1 domains) was constructed by site-directed mutagenesis (Kunkel, *supra*) using synthetic oligonucleotides to introduce the murine framework residues into 2H7.v2. The sequence of the resulting plasmid construct for expression of the chimeric Fab known as 2H7.v6.8, is shown in Fig. 3. Each encoded chain of the Fab has a 23 amino acid *St*II secretion signal sequence as described for pVX4 (Fig.2) above.

Based on a sequence comparison of the murine 2H7 framework residues with the human $V_\kappa I, V_H III$ consensus framework (Figures 1A and 1B) and previously humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285-4289 (1992)), several framework mutations were introduced into the 2H7.v2 Fab construct by site-directed mutagenesis. These mutations result in a change of certain human consensus framework residues to those found in the murine 2H7 framework, at sites that might affect CDR conformations or antigen contacts. Version 3 contained $V_H(R71V, N73K)$, version 4 contained $V_H(R71V)$, version 5 contained $V_H(R71V, N73K)$ and $V_L(L46P)$, and version 6 contained $V_H(R71V, N73K)$ and $V_L(L46P, L47W)$.

Humanized and chimeric Fab versions of m2H7 antibody were expressed in E. coli and purified as follows. Plasmids were transformed into E. coli strain XL-1 Blue (Stratagene, San Diego, CA) for preparation of double-and single-stranded DNA. For each variant, both light and heavy chains were completely sequenced using the dideoxynucleotide method (Sequenase, U.S. Biochemical Corp.). Plasmids were transformed into E. coli strain 16C9, a derivative of MM294, plated onto LB plates containing 5 μg/ml carbenicillin, and a single colony selected for protein expression. The single colony was grown in 5 ml LB-100 μg/ml carbenicillin for 5-8 h at 37° C. The 5 ml culture was added to 500 ml AP5-100 μg/ml carbenicillin and allowed to grow for 16 h in a 4 L baffled shake flask at 37°C. AP5 media consists of: 1.5g glucose, 11.0 Hycase SF, 0.6g yeast extract (certified), 0.19g anhydrous $MgSO_4$, 1.07g NH_4Cl, 3.73g KCl, 1.2g NaCl, 120 ml 1 M triethanolamine, pH 7.4, to 1 L water and then sterile filtered through 0.1 μm Sealkeen filter.

Cells were harvested by centrifugation in a 1 L centrifuge bottle (Nalgene) at 3000xg and the supernatant removed. After freezing for 1 h, the pellet was resuspended in 25 ml cold 10 mM MES-10 mM EDTA, pH 5.0 (buffer A). 250 μl of 0.1M PMSF (Sigma) was added to inhibit proteolysis and 3.5 ml of stock 10 mg/ml hen egg white lysozyme (Sigma) was added to aid lysis of the bacterial cell wall. After gentle shaking on ice for 1 h, the sample was centrifuged at 40,000xg for 15 min. The supernatant was brought to 50 ml with buffer A and loaded onto a 2 ml DEAE column equilibrated with buffer A. The flow-through was then applied to a protein G-Sepharose CL-4B (Pharmacia) column (0.5 ml bed volume) equilibrated with buffer A. The column was washed with 10 ml buffer A and eluted with 3 ml 0.3 M glycine, pH 3.0, into 1.25 ml 1 M Tris, pH 8.0. The F(ab) was then buffer exchanged into PBS using a Centricon-30 (Amicon) and concentrated to a final volume of 0.5 ml. SDS-PAGE gels of all F(ab)s were run to ascertain purity and the molecular weight of each variant was verified by electrospray mass spectrometry.

5   In cell-based ELISA binding assays (described below), the binding of Fabs, including chimeric 2H7
Fab, to CD20 was difficult to detect. Therefore, the 2H7 Fab versions were reformatted as full-length IgG1
antibodies for assays and further mutagenesis.

           Plasmids for expression of full-length IgG's were constructed by subcloning the V_L and V_H
domains of chimeric 2H7 (v6.8) Fab as well as humanized Fab versions 2 to 6 into previously described
10   pRK vectors for mammalian cell expression (Gorman et al.; *DNA Prot. Eng. Tech.* 2:3-10 (1990)). Briefly,
each Fab construct was digested with *EcoRV* and *Blp*I to excise a V_L fragment, which was cloned into the
*EcoRV/Blp*I sites of plasmid pDR1 (Fig. 4) for expression of the complete light chain (V_L-C_L domains).
Additionally, each Fab construct was digested with *Pvu*II and *Apa*I to excise a V_H fragment, which was
cloned into the *Pvu*II/*Apa*I sites of plasmid pDR2 (Fig. 5) for expression of the complete heavy chain (VH-
15   CH_1-hinge-CH_2-CH_3 domains). For each IgG variant, transient transfections were performed by
cotransfecting a light-chain expressing plasmid and a heavy-chain expressing plasmid into an adenovirus-
transformed human embryonic kidney cell line, 293 (Graham et al., *J. Gen. Virol.*, 36:59-74, (1977)).
Briefly, 293 cells were split on the day prior to transfection, and plated in serum-containing medium. On the
following day, double-stranded DNA prepared as a calcium phosphate precipitate was added, followed by
20   pAdVAntage™ DNA (Promega, Madison, WI), and cells were incubated overnight at 37°C. Cells were
cultured in serum-free medium and harvested after 4 days. Antibodies were purified from culture
supernatants using protein A-Sepharose CL-4B, then buffer exchanged into 10 mM sodium succinate, 140
mM NaCl, pH 6.0, and concentrated using a Centricon-10 (Amicon). Protein concentrations were
determined by quantitative amino acid analysis.

25   To measure relative binding affinities to the CD20 antigen, a cell-based ELISA assay was
developed. Human B-lymphoblastoid WIL2-S cells (ATCC CRL 8885, American Type Culture Collection,
Rockville, MD) were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 20 mM HEPES, pH 7.2
and 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 incubator. The cells were washed with
PBS containing 1% FBS (assay buffer) and seeded at 250-300,000 cell/well in 96-well round bottom plates
30   (Nunc, Roskilde, Denmark). Two-fold serially diluted standard (15.6-1000 ng/ml of 2H7 v6.8 chimeric IgG)
and threefold serially diluted samples (2.7-2000 ng/ml) in assay buffer were added to the plates. The plates
were buried in ice and incubated for 45 min. To remove the unbound antibody, 0.1 mL assay buffer were
added to the wells. Plates were centrifuged and supernatants were removed. Cells were washed two more
times with 0.2 mL assay buffer. Antibody bound to the plates was detected by adding peroxidase conjugated
35   goat anti-human Fc antibody (Jackson ImmunoResearch, West Grove, PA) to the plates. After a 45 min
incubation, cells were washed as described before. TMB substrate (3,3',5,5'-tetramethyl benzidine;
Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to the plates. The reaction was stopped by
adding 1 M phosphoric acid. Titration curves were fit with a four-parameter nonlinear regression curve-
fitting program (KaleidaGraph, Synergy software, Reading, PA). The absorbance at the midpoint of the
40   titration curve (mid-OD) and its corresponding concentration of the standard were determined. Then the
concentration of each variant at this mid-OD was determined, and the concentration of the standard was
divided by that of each variant. Hence the values are a ratio of the binding of each variant relative to the

5   standard. Standard deviations in relative affinity (equivalent concentration) were generally +/- 10% between

experiments.

As shown in Table 2, binding of the CDR-swap variant (v.2) was extremely reduced compared to

chimeric 2H7 (v.6.8). However, versions 3 to 6 showed improved binding. To determine the minimum

number of mutations that might be required to restore binding affinity to that of chimeric 2H7, additional

10   mutations and combinations of mutations were constructed by site-direct mutagenesis to produce variants 7

to 17 as indicated in Table 3. In particular, these included V_H mutations A49G, F67A, I69L, N73K, and

L78A; and V_L mutations M4L, M33I, and F71Y. Versions 16 and 17 showed the best relative binding

affinities, within 2-fold of that of the chimeric version, with no significant difference (s.d. = +/- 10%)

between the two. To minimize the number of mutations, version 16, having only 4 mutations of human

15   framework residues to murine framework residues (Table 3), was therefore chosen as the humanized form

for additional characterization.

Table 2. Relative binding affinity of humanized 2H7 IgG variants to CD20 compared to chimeric 2H7

using cell-based ELISA. The relative binding is expressed as the concentration of the chimeric 2H7 over the

20   concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker affinity for

the variant. Standard deviation in relative affinity determination averaged +/- 10%. Framework

substitutions in the variable domains are relative to the CDR-swap version according to the numbering

system of Kabat (Kabat et al., *supra*).

2H7 version	Heavy chain (V_H) substitutions	Light Chain (V_L) substitutions	Relative binding
6.8	(Chimera)	(Chimera)	-1-
2	(CDR swap)	(CDR swap)	0.01
3	R71V, N73K	(CDR swap)	0.21
4	R71V	(CDR swap)	0.21
5	R71V, N73K	L46P	0.50
6	R71V, N73K	L46P, L47W	0.58
7	R71V	L46P	0.33
8	R71V, L78A	L46P	0.19
9	R71V, F67A	L46P	0.07
10	R71V, F67A, I69L	L46P	0.12
11	R71V, F67A, L78A	L46P	0.19
12	R71V	L46P, M4L	0.32
13	R71V	L46P, M33I	0.31
14	R71V	L46P, F71Y	0.25
15	R71V	L46P, M4L, M33I	0.26
16	R71V, N73K, A49G	L46P	0.65
17	R71V, N73K, A49G	L46P, L47W	0.67

25

5   **Table 3** Oligonucleotide sequences used for construction of mutations VH(A49G, R71V, N73K) and

VL(L46P) in humanized 2H7 version 16 (2H7.v16). Underlined codons encode the indicated amino acid

substitutions. For V$_H$ (R71V, N73K) and V$_L$ (L46P), the oligos are shown as the sense strand since these

were used for mutagenesis on the Fab template, while for V$_H$ (A49G), the oligo is shown as the anti-sense

strand, since this was used with the pRK (IgG heavy chain) template. The protein sequence of version 16 is

10   shown in Fig. 6 and Fig. 7.

Substitution	Oligonucleotide sequence
V$_H$ (R71V, N73K)	GT TTC ACT ATA AGT <u>GTC</u> GAC <u>AAG</u> TCC AAA AAC ACA TT (SEQ ID NO. _)
V$_H$ (A49G)	GCCAGGATAGATGGCGCCAACCCATTCCAGGCC (SEQ ID NO. _)
V$_L$ (L46P)	AAGCTCCGAAACCACTGATTTACGCT (SEQ ID NO. _)

Example 2
Antigen-binding determinants (paratope) of 2H7

Alanine substitutions (Cunningham & Wells, *Science* 244: 1081-1085 (1989) were made in

15   2H7.v16 or 2H7.v17 in order to test the contributions of individual side chains of the antibody in binding to

CD20. IgG variants were expressed in 293 cells from pDR1 and pDR2 vectors, purified, and assayed for

relative binding affinity as described above. Several alanine substitutions resulted in significant decreases in

relative binding to CD20 on WIL-2S cells (Table 4).

20   Table 4. Effects of alanine substitutions in the CDR regions of humanized 2H7.v16 measured using cell-

based ELISA (WIL2-S cells). The relative binding is expressed as the concentration of the 2H7.v16 parent

over the concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker

affinity for the variant; a ratio >1 indicates higher affinity for the variant. Standard deviation in relative

affinity determination averaged +/- 10%. Framework substitutions in the variable domains are relative to

25   2H7.v16 according to the numbering system of Kabat (Kabat et al., *supra*). NBD means no detectable

binding. The two numbers for version 45 are from separate experiments.

2H7 version	CDR location	Heavy chain substitutions	Light chain substitutions	Relative binding
16	-	-	-	-1-
140	H1	G26A	-	0.63
141	H1	Y27A	-	0.47
34	H1	T28A	-	0.86
35	H1	F29A	-	0.07
36	H1	T30A	-	0.81
37	H1	S31A	-	0.97
142	H1	Y32A	-	0.63
143	H1	N33A	-	NDB
144	H1	M34A	-	1.2
145	H1	H35A	-	<0.25
146	H2	A50G	-	0.31
147	H2	I51A	-	0.65

38	H2	Y52A	-	0.01
148	H2	P52aA	-	0.66
39	H2	G53A	-	0.89
67	H2	N54A	-	1.4
40	H2	G55A	-	0.79
41	H2	D56A	-	2.0
89	H2	T57A	-	0.61
90	H2	S58A	-	0.92
91	H2	Y59A	-	0.74
92	H2	N60A	-	0.80
93	H2	Q61A	-	0.83
94	H2	K62A	-	0.44
95	H2	F63A	-	0.51
83	H2	V71A	-	0.96
149	H2	K64A	-	0.82
150	H2	G65A	-	1.2
153	H3	V95A	-	0.89
42	H3	V96A	-	0.98
43	H3	Y97A	-	0.63
44	H3	Y98A	-	0.40
45	H3	S99A	-	0.84; 0.92
46	H3	N100A	-	0.81
47	H3	S100aA	-	0.85
48	H3	Y100bA	-	0.78
49	H3	W100cA	-	0.02
59	H3	Y100dA	-	0.98
60	H3	F100eA	-	NDB
61	H3	D101A	-	0.31
151	H3	V102A	-	1.1
117	L1	-	R24A	0.85
118	L1	-	A25G	0.86
119	L1	-	S26A	0.98
120	L1	-	S27A	0.98
121	L1	-	S28A	1.0
122	L1	-	V29A	0.41
50	L1	-	S30A	0.96
51	L1	-	Y32A	1.0
123	L1	-	M33A	1.0
124	L1	-	H34A	0.21
125	L2	-	A50G	0.92
126	L2	-	P51A	0.88
52	L2	-	S52A	0.80
53	L2	-	N53A	0.76
54	L2	-	L54A	0.60
127	L2	-	A55G	1.1
128	L2	-	S56A	1.1

129	L3	-	Q89A	0.46
130	L3	-	Q90A	<0.22
55	L2	-	W91A	0.88
56	L3	-	S92A	1.1
57	L3	-	F93A	0.36
58	L3	-	N94A	0.61
131	L3	-	P95A	NDB
132	L3	-	P96A	0.18
133	L3	-	T97A	<0.22

Example 3

Additional mutations within 2H7 CDR regions

Substitutions of additional residues and combinations of substitutions at CDR positions that were identified as important by Ala-scanning were also tested. Several combination variants, particularly v.96 appeared to bind more tightly than v.16.

Table 5. Effects of combinations of mutations and non-alanine substitutions in the CDR regions of humanized 2H7.v16 measured using cell-based ELISA (WIL2-S cells). The relative binding to CD20 is expressed as the concentration of the 2H7.v16 parent over the concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker affinity for the variant; a ratio >1 indicates higher affinity for the variant. Standard deviation in relative affinity determination averaged +/- 10%. Framework substitutions in the variable domains are relative to 2H7.v16 according to the numbering system of Kabat (Kabat et al., *supra*).

2H7 version	Heavy chain substitutions	Light chain substitutions	Relative binding
16	-	-	-1-
96	D56A, N100A	S92A	3.5
97	S99T, N100G, Y100bI	-	0.99
98	S99G, N100S, Y100bI	-	1.6
99	N100G, Y100bI	-	0.80
101	N54S, D56A	-	1.7
102	N54K, D56A	-	0.48
103	D56A, N100A	-	2.1
104	S99T, N100G	-	0.81
105	S99G, N100S	-	1.1
106	N100G	-	~1
167	S100aG, Y100bS	-	
136	D56A, N100A	S56A, S92A	2.6
137	D56A, N100A	A55G, S92A	2.1
156	D56A, N100A	S26A, S56A, S92A	2.1
107	D56A, N100A, Y100bI	S92A	not expressed
182	Y27W	-	

183	Y27F	-	
184	F29Y	-	
185	F29W	-	
186	Y32F	-	
187	Y32W	-	
188	N33Q	-	
189	N33D	-	
190	N33Y	-	
191	N33S	-	
208	H35S	-	
209	A50S	-	
210	A50R	-	
211	A50V	-	
212	A50L	-	
168	Y52W	-	
169	Y52F	-	0.75
170	N54D	-	0.25
171	N54S	-	1.2
172	D56K	-	1
173	D56R	-	
174	D56H	-	1.5
175	D56E	-	1.2
213	D56S	-	
214	D56G	-	
215	D56N	-	
216	D56Y	-	
176	Y59W	-	
177	Y59F	-	
180	K62R	-	
181	K62D	-	
178	F63W	-	
179	F63Y	-	
157	Y97W	-	0.64
158	Y97F	-	1.2
159	Y98W	-	0.64
160	Y98F	-	0.88
106	N100G	-	
161	W100cY	-	0.05
162	W100cF	-	0.27
163	F100eY	-	0.59
164	F100eW	-	0.71
165	D101N	-	0.64
166	S99G, N100G, S100aD,Y100b deleted	-	0.99
217	V102Y	-	1.0
207	-	H34Y	

192	-	Q89E	
193	-	Q89N	
194	-	Q90E	
195	-	Q90N	
196	-	W91Y	
197	-	W91F	
205	-	S92N	
206	-	S92G	
198	-	F93Y	
199	-	F93W	
204	-	F93S, N94Y	
200	-	P96L	
201	-	P96Y	
202	-	P96W	
203	-	P96R	

Example 4
Mutations at sites of framework humanization substitutions

Substitutions of additional residues at framework positions that were changed during humanization were also tested in the 2H7.v16 background. In particular, alternative framework substitutions that were neither found in the murine 2H7 parent nor the human consensus framework were made at V_L(P46) and V_H(G49, V71, and K73).

These substitutions generally led to little change in relative binding (Table 6), indicating that there is some flexibility in framework residues at these positions.

Table 6. Relative binding in a cell-based (WIL2-S) assay of framework substitutions. IgG variants are shown with mutations with respect to the 2H7.v16 background. The relative binding is expressed as the concentration of the 2H7.v6.8 chimera over the concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker affinity for the variant; a ratio >1 indicates higher affinity for the variant. Standard deviation in relative affinity determination averaged +/- 10%. Framework substitutions in the variable domains are relative to 2H7.v16 according to the numbering system of Kabat (Kabat et al., *supra*). (*) Variants that were assayed with 2H7.v16 as the standard comparator; relative values are normalized to that of the chimera.

2H7 version	Heavy chain substitutions	Light chain substitutions	Relative binding
6.8	(chimera)	(chimera)	-1-
16	-	-	0.64
78	K73R	-	0.72
79	K73H	-	0.49
80	K73Q	-	0.58
81	V71I	-	0.42
82	V71T	-	0.58
83	V71A	-	
84	G49S	-	0.32

85	G49L	-	
86	-	P46E	0.22
87	-	P46V	0.51
88	-	P46T	
108	G49A, V71T, K73R	S92A, M32L, P46T	0.026*
109	G49A, A49G, V71T, K73R	S92A, M32L, P46T	0.026*
110	K73R, D56A, N100A	S92A, M32L	Not expressed
111	G49A, V71T, K73R	-	0.46*
112	G49A, A50G, V71T, K73R	-	0.12*

(*) Variants that were assayed with 2H7.v16 as the standard comparator;
relative values are normalized to that of the chimera.

Example 5

Humanized 2H7 variants with enhanced effector functions

Because 2H7 can mediate lysis of B-cells through both complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), we sought to produce variants of humanized 2H7.v16 with improved CDC and ADCC activity. Mutations of certain residues within the Fc regions of other antibodies have been described (Idusogie et al., *J. Immunol.* 166:2571-2575 (2001)) for improving CDC through enhanced binding to the complement component C1q. Mutations have also been described (Shields et al., *J. Biol. Chem.* 276:6591-6604 (2001); Presta et al., *Biochem. Soc. Trans.* 30:487-490 (2002)) for improving ADCC through enhanced IgG binding to activating Fcγ receptors and reduced IgG binding to inhibitory Fcγ receptors. In particular, three mutations have been identified for improving CDC and ADCC activity: S298A/E333A/K334A (also referred to herein as a triple Ala mutant or variant; numbering in the Fc region is according to the EU numbering system; Kabat et al., *supra*) as described (Idusogie et al., *supra* (2001); Shields et al., *supra*).

In order to enhance CDC and ADCC activity of 2H7, a triple Ala mutant of the 2H7 Fc was constructed. A humanized variant of the anti-HER2 antibody 4d5 has been produced with mutations S298A/E333A/K334A and is known as 4D5Fc110 (*i.e.*, anti-p[185]HER2 IgG1 (S298A/E333A/K334A); Shields et al., *supra*). A plasmid, p4D5Fc110 encoding antibody 4D5Fc110 (Shields et al., *supra*) was digested with *Apa*I and *Hind*III, and the Fc-fragment (containing mutations S298A/E333A/K334A) was ligated into the *Apa*I/*Hind*III sites of the 2H7 heavy-chain vector pDR2-v16, to produce pDR2-v31. The amino acid sequence of the version 31 complete H chain is shown in Fig. 8. The L chain is the same as that of v16.

Although the constant domains of the Fc region of IgG1 antibodies are relatively conserved within a given species, allelic variations exist (reviewed by Lefranc and Lefranc, in *The human IgG subclasses: molecular analysis of structure, function, and regulation*, pp. 43-78, F. Shakib (ed.), Pergammon Press, Oxford (1990)).

Table 7. Effects of substitutions in the Fc region on CD20 binding. Relative binding to CD20 was measured in a cell-based (WIL2-S) assay of framework substitutions. Fc mutations (*) are indicated by EU numbering (Kabat, *supra*) and are relative to the 2H7.v16 parent. The combination of three Ala changes in

the Fc region of v.31 is described as "Fc110." IgG variants are shown with mutations with respect to the 2H7.v16 background. The relative binding is expressed as the concentration of the 2H7.v6.8 chimera over the concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker affinity for the variant. Standard deviation in relative affinity determination averaged +/- 10%.

2H7 version	Fc Substitutions*	Relative binding
6.8	–	-1-
16	–	0.65
31	S298A, E333A, K334A	0.62

Example 6

Humanized 2H7 variants with enhanced stability

For development as therapeutic proteins, it is desirable to choose variants that remain stable with respect to oxidation, deamidation, or other processes that may affect product quality, in a suitable formulation buffer. In 2H7.v16, several residues were identified as possible sources of instability: VL (M32) and VH (M34, N100). Therefore, mutations were introduced at these sites for comparison with v16.

Table 8. Relative binding of 2H7 variants designed for enhanced stability and/or effector function, to CD20 in a cell-based (WIL2-S) assay. IgG variants are shown with mutations with respect to the 2H7.v16 background. The relative binding is expressed as the concentration of the 2H7.v6.8 chimera over the concentration of the variant required for equivalent binding; hence a ratio <1 indicates weaker affinity for the variant. Standard deviation in relative affinity determination averaged +/- 10%. Framework substitutions in the variable domains are relative to 2H7.v16 according to the numbering system of Kabat and Fc mutations (*) are indicated by EU numbering (Kabat et al., *supra*). (**) Variants that were measured with 2H7.v16 as the standard comparator; relative values are normalized to that of the chimera.

Additional Fc mutations were combined with stability or affinity-enhancing mutations to alter or enhance effector functions based on previously reported mutations (Idusogie et al. (2000); Idusogie et al. (2001); Shields et al. (2001)). These changes include S298, E333A, K334A as described in Example 5; K322A to reduced CDC activity; D265A to reduce ADCC activity; K326A or K326W to enhance CDC activity; and E356D/M358L to test the effects of allotypic changes in the Fc region. None of these mutations caused significant differences in CD20 binding affinity.

2H7 version	Heavy chain (V$_H$) changes	Light chain (V$_L$) changes	Fc changes *	Relative binding
6.8	(chimera)	(chimera)	–	-1-
16	–	–	–	0.65
62	–	M32I	–	0.46
63	M34I	–	–	0.49
64	N100A	–	–	
65	N100A	L47W	–	0.74
66	S99A	L47W	–	0.62
67	N54A	–	–	

68	-	M32I	-	0.48
69	-	M32L	-	0.52
70	N100A	-	S298A, E333A, K334A	0.80
71	N100D	-	S298A, E333A, K334A	0.44
72	N100A	M32I	-	0.58
73	N100A	M32L	-	0.53
74	N100A	M32I	S298A, E333A, K334A	0.61
75	N100A	M32L	S298A, E333A, K334A	0.60
113	-	-	E356D, M358L	0.60**
114	D56A, N100A	M32L, S92A	S298A, E333A, K334A	1.2**
115	D56A, N100A	M32L, S92A	S298A, E333A, K334A, E356D, M358L	1.4**
116	D56A, N100A	M32L, S92A	S298A, K334A, K322A	1.2**
134	D56A, N100A	M32L, S92A	E356D, M358L, D265A	1.5**
135	D56A, N100A	M32L, S92A	E356D, M358L, D265A, K326W	0.95**
138	D56A, N100A	M32L, S92A	S298A, E333A, K334A, K326A	1.2**
139	D56A, N100A	M32L, S92A	S298A, E333A, K334A, K326A, E356N, M358L	1.1**
154	-	-	D265A	0.70**
155	-	-	S298A, K322A, K334A	0.70**

(**) Variants that were measured with 2H7.v16 as comparator;
relative binding values are normalized to that of the chimera.

To test the effects of stability mutations on the rate of protein degradation, 2H7.v16 and 2H7.v73 were formulated at 12-14 mg/mL in 10 mM histidine, 6% sucrose, 0.02% polysorbate 20, pH 5.8 and incubated at 40°C for 16 days. The incubated samples were then assayed for changes in charge variants by ion exchange chromatography, aggregation and fragmentation by size exclusion chromatography, and relative binding by testing in a cell-based (WIL2-S) assay.

The results (Fig. 9) show that 2H7 v.73 has greater stability compared to 2H7 v.16 with respect to losses in the fraction of main peak by ion exchange chromatography under accelerated stability conditions. No significant differences were seen with respect to aggregation, fragmentation, or binding affinity.

Example 7
Scatchard analysis of antibody binding to CD20 on WIL2-S cells

Equilibrium dissociation constants (K_d) were determined for 2H7 IgG variants binding to WIL2-S cells using radiolabeled 2H7 IgG. IgG variants were produced in CHO cells. Rituxan® (source for all experiments is Genentech, S. San Francisco, CA) and murine 2H7 (BD PharMingen, San Diego, CA) were used for comparison with humanized variants. The murine 2H7 antibody is also available from other sources, e.g., eBioscience, and Calbiochem (both of San Diego, CA), Accurate Chemical & Scientific Corp., (Westbury, NY), Ancell (Bayport, MN), and Vinci-Biochem (Vinci, Italy). All dilutions were performed in binding assay buffer (DMEM media containing 1% bovine serum albumin, 25 mM HEPES pH 7.2, and 0.01% sodium azide). Aliquots (0.025 mL) of ^{125}I-2H7.v16 (iodinated with lactoperoxidase) at a concentration of 0.8 nM were dispensed into wells of a V-bottom 96-well microassay plate, and serial dilutions (0.05 mL) of cold antibody were added and mixed. WIL2-S cells (60,000 cells in 0.025 mL) were then added. The plate was sealed and incubated at room temperature for 24h, then centrifuged for 15 min at 3,500 RPM. The supernatant was then aspirated and the cell pellet was washed and centrifuged. The

5   supernatant was again aspirated, and the pellets were dissolved in 1N NaOH and transferred to tubes for

gamma counting. The data were used for Scatchard analysis (Munson and Rodbard, *Anal. Biochem.*

107:220-239 (1980)) using the program Ligand (McPherson, *Comput. Programs Biomed.* 17: 107-114

(1983)). The results, shown in Table 9, indicate that humanized 2H7 variants had similar CD20 binding

affinity as compared to murine 2H7, and similar binding affinity to Rituxan®. It is expected that 2H7.v31

10   will have very similar K_d to v.16 on the basis of the binding shown in Table 7 above.

Table 9. Equilibrium binding affinity of 2H7 variants from Scatchard analysis

Antibody variant	K_d (nM)	n
Rituxan	0.99±0.49	3
2H7 (murine)	1.23±0.29	3
2H7.v16	0.84±0.37	4
2H7.v73	1.22±0.39	4
2H7.v75	1.09±0.17	4

Example 8
Complement Dependent Cytotoxicity (CDC) Assays

15

2H7 IgG variants were assayed for their ability to mediate complement-dependent lysis of WIL2-S

cells, a CD20 expressing lymphoblastoid B-cell line, essentially as described (Idusogie et al., *J. Immunol.*

164:4178-4184 (2000); Idusogie et al., *J. Immunol.* 166:2571-2575 (2001)). Antibodies were serially diluted

1:3 from a 0.1 mg/mL stock solution. A 0.05 mL aliquot of each dilution was added to a 96-well tissue

20   culture plate that contained 0.05 mL of a solution of normal human complement (Quidel, San Diego, CA)

To this mixture, 50,000 WIL2-S cells were added in a 0.05 mL volume. After incubation for 2h at 37°C,

0.05 mL of a solution of Alamar blue (Accumed International, Westlake, OH) was added, and incubation

was continued for an additional 18h at 37°C. Covers were then removed from the plates, and they were

shaken for 15 min at room temperature on an orbital shaker. Relative fluorescent units (RFU) were read

25   using a 530 nm excitation filter and a 590 nm emission filter. An EC_{50} was calculated by fitting RFU as a

function of concentration for each antibody using KaleidaGraph software.

The results (Table 10) show surprising improvement in CDC by humanized 2H7 antibodies, with

relative potency similar to Rituxan® for v.73, 3-fold more potent than Rituxan® for v.75, and 3-fold weaker

than Rituxan® for v.16.

30   **Table 10.** CDC activity of 2H7 antibodies compared to Rituxan. Numbers >1 indicate less potent CDC

activity than Rituxan® and numbers <1 indicate more potent activity than Rituxan®. Antibodies were

produced from stable CHO lines, except that those indicated by (*) were produced transiently .

Antibody variant	n	EC_{50}(variant)/EC_{50}(Rituxan)
Rituxan®	4	-1-
2H7.v16	4	3.72; 4.08
2H7.v31*	4	2.21
2H7.v73	4	1.05
2H7.v75	4	0.33

2H7.v96*	4	0.956
2H7.v114*	4	0.378
2H7.v115*	4	0.475
2H7.v116*	1	>100
2H7.v135*	2	0.42

Example 9
Antibody Dependent Cellular Cytotoxicity (ADCC) Assays

2H7 IgG variants were assayed for their ability to mediate Natural-Killer cell (NK cell) lysis of WIL2-S cells, a CD20 expressing lymphoblastoid B-cell line, essentially as described (Shields et al., *J. Biol. Chem.* 276:6591-6604 (2001)) using a lactate dehydrogenase (LDH) readout. NK cells were prepared from 100 mL of heparinized blood, diluted with 100 mL of PBS (phosphate buffered saline), obtained from normal human donors who had been isotyped for FcγRIII, also known as CD16 (Koene et al., *Blood* 90:1109-1114 (1997)). In this experiment, the NK cells were from human donors heterozygous for CD16 (F158/V158). The diluted blood was layered over 15 mL of lymphocyte separation medium (ICN Biochemical, Aurora, Ohio) and centrifuged for 20 min at 2000 RPM. White cells at the interface between layers were dispensed to 4 clean 50-mL tubes, which were filled with RPMI medium containing 15% fetal calf serum. Tubes were centrifuged for 5 min at 1400 RPM and the supernatant discarded. Pellets were resuspended in MACS buffer (0.5% BSA, 2mM EDTA), and NK cells were purified using beads (NK Cell Isolation Kit, 130-046-502) according to the manufacturer's protocol (Miltenyi Biotech,). NK cells were diluted in MACS buffer to 2×10^6 cells/mL.

Serial dilutions of antibody (0.05 mL) in assay medium (F12/DMEM 50:50 without glycine, 1 mM HEPES buffer pH 7.2, Pennicillin/Streptomycin (100 units/mL; Gibco), glutamine, and 1% heat-inactivated fetal bovine serum) were added to a 96-well round-bottom tissue culture plate. WIL2-S cells were diluted in assay buffer to a concentration of 4×10^5/mL. WIL2-S cells (0.05 mL per well) were mixed with diluted antibody in the 96-well plate and incubated for 30 min at room temperature to allow binding of antibody to CD20 (opsonization).

The ADCC reaction was initiated by adding 0.1 mL of NK cells to each well. In control wells, 2% Triton X-100 was added. The plate was then incubated for 4h at 37°C. Levels of LDH released were measured using a cytotoxicity (LDH) detection kit (Kit#1644793, Roche Diagnostics, Indianapolis, Indiana.) following the manufacturers instructions. 0.1 mL of LDH developer was added to each well, followed by mixing for 10s. The plate was then covered with aluminum foil and incubated in the dark at room temperature for 15 min. Optical density at 490 nm was then read and use to calculate % lysis by dividing by the total LDH measured in control wells. Lysis was plotted as a function of antibody concentration, and a 4-parameter curve fit (KaleidaGraph) was used to determine EC_{50} concentrations.

The results showed that humanized 2H7 antibodies were active in ADCC, with relative potency 20-fold higher than Rituxan® for v.31 and v.75, 5-fold more potent than Rituxan® for v.16, and almost 4-fold higher than Rituxan® for v.73.

5   **Table 11.** ADCC activity of 2H7 antibodies on WIL2-S cells compared to 2H7.v16, based on n
experiments. (Values >1 indicate lower potency than 2H7.v16, and values <1 indicate greater potency.)

Antibody variant	n	EC_{50}(variant)/EC_{50}(2H7.v16)
Rituxan®	4	5.3
2H7.v16	5	1
2H7.v31	1	0.24
2H7.v73	5	1.4
2H7.v75	4	0.25

Additional ADCC assays were carried out to compare combination-variants of 2H7 with Rituxan®.
10   The results of these assays indicated that 2H7.v114 and 2H7.v115 have >10-fold improved ADCC potency
as compared to Rituxan® (Table 12).

Table 12. ADCC activity of 2H7 antibodies on WIL2-S cells compared to Rituxan®, based on n
experiments (Values >1 indicate lower potency than Rituxan®, and values <1 indicate greater potency).

15

Antibody variant		EC50(variant)/EC50(Rituxan)
Rituxan®	2	-1-
2H7 v.16	2	0.52
2H7 v.96	2	0.58
2H7.v114	2	0.093
2H7.v115	2	0.083
2H7.v116	2	0.30

Example 10

In vivo effects of 2H7 variants in a pilot study in cynomolgus monkeys

2H7 variants, produced by transient transfection of CHO cells, were tested in normal male
20   cynomolgus (*Macaca fascicularis*) monkeys in order to evaluate their *in vivo* activities. Other anti-CD20
antibodies, such as C2B8 (Rituxan®) have demonstrated an ability to deplete B-cells in normal primates
(Reff et al., *Blood* 83: 435-445 (1994)).

In one study, humanized 2H7 variants were compared. In a parallel study, Rituxan® was also
tested in cynomolgus monkeys. Four monkeys were used in each of five dose groups: (1) vehicle, (2) 0.05
25   mg/kg hu2H7.v16, (3) 10 mg/kg hu2H7.v16, (4) 0.05 mg/kg hu2H7.v31, and (5) 10 mg/kg hu2H7.v31.
Antibodies were administered intravenously at a concentration of 0, 0.2, or 20 mg/mL, for a total of two
doses, one on day 1 of the study, and another on day 8. The first day of dosing is designated day 1 and the
previous day is designated day –1; the first day of recovery (for 2 animals in each group) is designated as
day 11. Blood samples were collected on days –19, -12, 1 (prior to dosing), and at 6h, 24h, and 72h
30   following the first dose. Additional samples were taken on day 8 (prior to dosing), day 10 (prior to sacrifice
of 2 animals/group), and on days 36 and 67 (for recovery animals).

Peripheral B-cell concentrations were determined by a FACS method that counted CD3-/CD40+
cells. The percent of CD3-CD40+ B cells of total lymphocytes in monkey samples were obtained by the
following gating strategy. The lymphocyte population was marked on the forward scatter/ side scatter

5   scattergram to define Region 1 (R1). Using events in R1, fluorescence intensity dot plots were displayed for
       CD40 and CD3 markers. Fluorescently labeled isotype controls were used to determine respective cutoff
       points for CD40 and CD3 positivity.

       The results indicated that both 2H7.v16 and 2H7.v31 were capable of producing full peripheral B-
       cell depletion at the 10 mg/kg dose and partial peripheral B-cell depletion at the 0.05 mg/kg dose (Fig. 11).
10   The time course and extent of B-cell depletion measured during the first 72h of dosing were similar for the
       two antibodies. Subsequent analysis of the recovery animals indicated that animals treated with 2H7.v31
       showed a prolonged depletion of B-cells as compared to those dosed with 2H7.v16. In particular, recovery
       animals treated with 10 mg/kg 2H7.v16, B-cells showed substantial B-cell recovery at some time between
       sampling on Day 10 and on Day 36. However, for recovery animals treated with 10 mg/kg 2H7.v31, B-cells
15   did not show recovery until some time between Day 36 and Day 67 (Fig. 11). This suggests a greater
       duration of full depletion by about one month for 2H7.v31 compared to 2H7.v16.

       No toxicity was observed in the monkey study at low or high dose and the gross pathology was
       normal. In other studies, v16 was well tolerated up to the highest dose evaluated of (100mg/kgx2 = 1200
       mg/m^2 x2) following i.v. administration of 2 doses given 2 weeks apart in these monkeys.
20   Data in Cynomolgus monkeys with 2H7.v16 versus Rituxan® suggests that a 5-fold reduction in
       CDC activity does not adversely affect potency. An antibody with potent ADCC activity but reduced CDC
       activity may have more favorable safety profile with regard to first infusion reactions than one with greater
       CDC activity.

25   **Example 11**
                 Fucose deficient 2H7 variant antibodies with enhanced effector function
       Normal CHO and HEK293 cells add fucose to IgG oligosaccharide to a high degree (97-98%). IgG
       from sera are also highly fucosylated.

       DP12, a dihydrofolate reductase minus (DHFR⁻) CHO cell line that is fucosylation competent, and
30   Lec13, a cell line that is deficient in protein fucosylation were used to produce antibodies for this study. The
       CHO cell line Pro-Lec13.6a (Lec13), was obtained from Professor Pamela Stanley of Albert Einstein
       College of Medicine of Yeshiva University. Parental lines are Pro- (proline auxotroph) and Gat- (glycine,
       adenosine, thymidine auxotroph). The CHO-DP12 cell line is a derivative of the CHO-K1 cell line (ATCC
       #CCL-61), which is dihydrofolate reductase deficient, and has a reduced requirement for insulin. Cell lines
35   were transfected with cDNA using the Superfect method (Qiagen, Valencia, CA). Selection of the Lec13
       cells expressing transfected antibodies was performed using puromycin dihydrochloride (Calbiochem, San
       Diego, CA) at 10 µg/ml in growth medium containing: MEM Alpha Medium with L-glutamine,
       ribonucleosides and deoxyribonucleosides (GIBCO-BRL, Gaithersburg, MD), supplemented with 10%
       inactivated FBS (GIBCO), 10 mM HEPES, and 1X penicillin/streptomycin (GIBCO). The CHO cells were
40   similarly selected in growth medium containing Ham's F12 without GHT: Low Glucose DMEM without
       Glycine with NaHCO3 supplemented with 5% FBS (GIBCO), 10 mM HEPES, 2 mM L-glutamine, 1X
       GHT(glycine, hypoxanthine,thymidine), and 1X penicillin/streptomycin.

       Colonies formed within two to three weeks and were pooled for expansion and protein expression.
       The cell pools were seeded initially at 3 x 10^6 cells/10 cm plate for small batch protein expression. The cells

5   were converted to serum-free media once they grew to 90-95% confluency and after 3-5 days cell

supernatants were collected and tested in an Fc IgG- and intact IgG-ELISA to estimate protein expression

levels. Lec13 and CHO cells were seeded at approximately 8×10^6 cells/15 cm plate one day prior to

converting to PS24 production medium, supplemented with 10 mg/L recombinant human insulin and 1 mg/L

trace elements.

10   Lec13 cells and DP12 cells remained in serum-free production medium for 3-5 days. Supernatants

were collected and clarified by centrifugation in 150 ml conical tubes to remove cells and debris. The

protease inhibitors PMSF and aprotinin (Sigma, St. Louis, MO) were added and the supernatants were

concentrated 5-fold on stirred cells using MWCO30 filters (Amicon, Beverly, MA) prior to immediate

purification using protein G chromatography (Amersham Pharmacia Biotech, Piscataway, NJ)). All proteins

15   were buffer exchanged into phosphate-buffered saline (PBS) using Centripriep-30 concentrators (Amicon)

and analyzed by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined using

A280 and verified using amino acid composition analysis.

The CHO cells were transfected with vectors expressing humanized 2H7v16, 2H7v.31 and selected

as described. The 2H7v.16 antibody retains the wild type Fc region while v.31 (see Example 5, Table 7

20   above) has an Fc region wherein 3 amino acid changes were made (S298A, E333A, K334A) which results in

higher affinity for the FcγRIIIa receptor (Shields et al. J. Biol. Chem. 276 (9):6591-6604 (2001)). Following

transfection and selection, individual colonies of cells were isolated and evaluated for protein expression

level and the highest producers were subjected to methotrexate selection to select for cells that had amplified

the plasmid copy number and which therefore produced higher levels of antibody. Cells were grown,

25   transferred to serum free medium for a period of 7 days, then the medium was collected, loaded onto a

protein A column and the antibody was eluted using standard techniques. The final concentration of the

antibody was determined using an Elisa that measures intact antibody. All proteins were buffer exchanged

into phosphate-buffered saline (PBS) using Centripriep-30 concentrators. (Amicon) and analyzed by SDS-

polyacrylamide gel electrophoresis.

30   *Matrix-Assisted Laser Desorption/Ionization Time-of-flight (MALDI-TOF) Mass Spectral Analysis*

of Asparagine-Linked Oligosaccharides: N-linked oligosaccharides were released from recombinant

glycoproteins using the procedure of Papac *et al.*, *Glycobiology* 8, 445-454 (1998). Briefly, the wells of a 96

well PVDF-lined microtitre plate (Millipore, Bedford, MA) were conditioned with 100 μl methanol that was

drawn through the PDVF membranes by applying vacuum to the Millipore Multiscreen vacuum manifold.

35   The conditioned PVDF membranes were washed with 3 X 250 μl water. Between all wash steps the wells

were drained completely by applying gentle vacuum to the manifold. The membranes were washed with

reduction and carboxymethylation buffer (RCM) consisting of 6 M guanidine hydrochloride, 360 mM Tris, 2

mM EDTA, pH 8.6. Glycoprotein samples (50 μg) were applied to individual wells, again drawn through the

PVDF membranes by gentle vacuum and the wells were washed with 2 X 50 μl of RCM buffer. The

40   immobilized samples were reduced by adding 50 μl of a 0.1 M dithiothreitol (DTT) solution to each well and

incubating the microtitre plate at 37°C for 1 hr. DTT was removed by vacuum and the wells were washed 4

x 250 μl water. Cysteine residues were carboxylmethylated by the addition of 50 μl of a 0.1 M iodoacetic

acid (IAA) solution which was freshly prepared in 1 M NaOH and diluted to 0.1 M with RCM buffer.

Carboxymethylation was accomplished by incubation for 30 min in the dark at ambient temperature.

5   Vacuum was applied to the plate to remove the IAA solution and the wells were washed with 4 x 250 μl
      purified water. The PVDF membranes were blocked by the addition of 100 μl of 1% PVP360
      (polyvinylpyrrolidine 360,000 MW) (Sigma) solution and incubation for 1 hr at ambient temperature. The
      PVP-360 solution was removed by gentle vacuum and the wells were washed 4 x 250 μl water. The PNGase
      F (New England Biolabs, Beverly, MA) digest solution, 25 μl of a 25 Unit/ml solution in 10 mM Tris

10   acetate, pH 8.4, was added to each well and the digest proceeded for 3 hr at 37°C. After digestion, the
      samples were transferred to 500 μl Eppendorf tubes and 2.5 μlL of a 1.5 M acetic acid solution was added to
      each sample. The acidified samples were incubated for 3 hr at ambient temperature to convert the
      oligosaccharides from glycosylamines to the hydroxyl form. Prior to MALDI-TOF mass spectral analysis,
      the released oligosaccharides were desalted using a 0.7-ml bed of cation exchange resin (AG50W-X8 resin

15   in the hydrogen form) (Bio-Rad, Hercules, CA) slurried packed into compact reaction tubes (US
      Biochemical, Cleveland, OH).

          For MALDI-TOF mass spectral analysis of the samples in the positive mode, the desalted
      oligosaccharides (0.5 μl aliquots) were applied to the stainless target with 0.5 μl of the 2,5 dihydroxybenzoic
      acid matrix (sDHB) that was prepared by dissolving 2 mg 2,5 dihydroxybenzoic acid with 0.1 mg of 5-

20   methoxyslicylic acid in 1 ml of ethanol/10 mM sodium chloride 1:1 (v/v). The sample/matrix mixture was
      dried by vacuum. For analysis in the negative mode, the desalted N-linked oligosaccharides (0.5 μl aliquots)
      were applied to the stainless target along with 0.5 μl 2',4',6'-trihydroxyacetophenone matrix (THAP)
      prepared in 1:3 (v/v) acetonitrile/13.3 mM ammonium citrate buffer. The sample/matrix mixture was
      vacuum dried and then allowed to absorb atmospheric moisture prior to analysis. Released oligosaccharides

25   were analyzed by MALDI-TOF on a PerSeptive BioSystems Voyager-DE mass spectrometer. The mass
      spectrometer was operated at 20 kV either in the positive or negative mode with the linear configuration and
      utilizing delayed extraction. Data were acquired using a laser power of 1300 and in the data summation
      mode (240 scans) to improve the signal to noise. The instrument was calibrated with a mixture of standard
      oligosaccharides and the data was smoothed using a 19 point Savitsky-Golay algorithm before the masses

30   were assigned. Integration of the mass spectral data was achieved using Caesar 7.0 data analysis software
      package (SciBridge Software).

      Natural killer (NK) cell antibody dependent cytoxicity assays.

          ADCC assays were performed as described in Example 9. NK to target cell (WIL2-S) ratio was 4
      to 1, assays were run for 4 hours, and toxicity was measured as before using lactose dehydrogenase assay.

35   Target cells were opsonized with the concentrations of antibody indicated for 30 min prior to addition of NK
      cells. The Rituxan® antibody used was from Genentech (S. San Francisco, CA). *Figure 12*shows the results
      of a representative ADCC assay.

          The results show that underfucosylated antbodies mediate NK cell target cell killing more
      efficiently than do antibodies with a full complement of fucose. The underfucosylated antibody, 2H7v.31, is

40   most efficient at mediating target cell killing. This antibody is effective at lower concentrations and is
      capable of mediating killing of a greater percentage of target cells at higher concentrations than are the other
      antibodies. The activity of the antibodies is as follows: Lec13-derived 2H7 v31> Lec 13 derived 2H7v16>
      Dp12 derived 2H7v31> Dp12 derived 2H7v16 > or = to Rituxan. The protein and carbohydrate alterations
      are additive. Comparison of the carbohydrate found on native IgG from the Lec13-produced and CHO-

5   produced IgG showed no appreciable differences in the extent of galactosylation and hence the results can be
       attributed solely to the presence/absence of fucose.

Example 12

Fucose-deficient 2H7 variant antibodies with enhanced ADCC in vivo

10   This example describes ADCC activity in vivo of the fucose-deficient humanized 2H7 variants

       including v.16 and v.31 produced in Lec13 compared to normal fucosylated counterparts produced in DP12,

       in mice expressing human CD16 [FcRγIII] and human CD20.

       Generation of huCD20Tg$^+$ huCD16Tg$^+$ mCD16$^{-/-}$ mice

              Human CD20 transgenic mice were generated from human CD20 BAC DNA (Invitrogen, Carlsbad,

15   CA). Mice were screened based on the FACS analysis of human CD20 expression. HuCD20 Tg$^+$ mice were

       then crossed with huCD16Tg$^+$mCD16$^{-/-}$ mice to generate huCD20Tg$^+$huCD16Tg$^+$mCD16$^{-/-}$ mice.

       In vivo treatment

              Ten to 100 μg of each of the 2H7 variants or Rituxan® is administrated to

       huCD20Tg$^+$huCD16Tg$^+$mCD16$^{-/-}$ mice via intraperitoneal injections. Equal amount of isotype-matched

20   antibodies will be applied similarly to the negative control group of animals.

       Mouse lymphocytes preparation

              Mouse lymphocytes from whole blood, spleen, lymph nodes and bone marrow are prepared

       according to standard protocol described in "Current Protocols in Immunology, edited by John Coligan, Ada

       Kruisbeek, David Margulies, Ethan Shevach and Warren Strober, 1994".

25   *FACS analysis*

              Half million cells are washed and resuspended in 100 μl of FACS buffer, which is phosphate

       buffered saline with 1% BSA, containing 5 μl of staining or control antibody. All the staining antibodies,

       including isotype controls, are obtained from PharMingen, San Diego, CA. Human CD20 expression is

       assessed by staining with Rituxan® along with FITC-conjugated anti-human IgG1 secondary antibody.

30   FACS analysis is conducted using FACScan and Cell Quest (Becton Dickinson Immunocytometry Systems,

       San Jose, CA). All the lymphocytes are defined in the forward and side light scatterings, while all the B

       lymphocytes are defined with the expression of B220 on the cell surface.

              B cell depletion and recovery are assessed by analyzing peripheral B cell counts and analysis of

       hCD20+ B cells by FACS in the spleen, lymph node and bone marrow on a daily basis for the first week

35   after injection and thereafter on a weekly basis. Serum levels of the injected 2H7 variant antibody are

       monitored.

              The results of this in vivo assay confirms the in vitro findings on the increased ADCC activity and

       greater B cell depletion of fucose-deficient 2H7 variants over wild-type (with resepct to fucosylation)

       glycosylation counterparts.

40

Example 13

Apoptosis Activity

Anti-CD20 antibodies including Rituxan® have been shown to induce *apoptosis in vitro* when crosslinked by a secondary antibody or by chemical means (Shan et al., Blood 9:1644-1652 (1998); Byrd et al., Blood 99:1038-43 (2002); Pederson et al., Blood 99:1314-19 (2002)). When chemically crosslinked, murine 2H7 dimers induced apoptosis of Daudi cells (Ghetie et al., Proc Natl Acad Sci USA 94:7509-14 (1997)). Crosslinking with a secondary antibody also induced apoptosis with the murine 2H7 antibody (Shan et al., 1998). These activities are believed to be physiologically relevant because a variety of mechanisms could lead to crosslinking of anti-CD20 antibodies bound to cell-surface CD20 *in vivo*.

RhuMAb 2H7.v16 [humanized 2H7 v16; RhuMAb stands for recombinant human monoclonal antibody] and Rituxan® were compared in apoptosis assays *in vitro* using a secondary crosslinking antibody. Ramos cells (CRL-1596, ATCC, Manassas, VA), a CD20-expressing, human B lymphocyte cell line, were used to measure the ability of the anti-CD20 monoclonal antibodies rhuMAb 2H7.v16 and Rituximab versus a negative-control antibody, Trastuzumab (Herceptin®, Genentech, South San Francisco, CA), to induce apoptosis as measured through Annexin V staining and propidium iodide dye exclusion (Vybrant® Apoptosis Assay Kit, Molecular Probes, Seattle, WA). The Ramos cells were cultured in RPMI-1640 medium (Gibco, Rockville, MD) containing 10% fetal bovine serum (Biosource International, Camarillo, CA) and 2 mM L-glutamine (Gibco). Prior to being assayed, the cells were washed twice in fresh media and then adjusted to a cell concentration of 2×10^6 per mL. Cells (150 µL) were added to 96-well assay plates (Becton Dickinson, Palo Alto, CA) which contained 150 µL of a predetermined amount of control IgG1, rhuMAb 2H7.v16, or Rituximab, along with F(ab)'2 goat anti-human Fc (Pierce Biotechnology, Rockford, IL). The final IgG concentrations were 100, 10, 1.0, 0.1, 0.01 and 0.001 nM, and the F(ab)'2 goat anti-human Fc antibody concentration was set at twice the respective sample antibody concentration. Each dilution was set up in triplicate. After a 24-hour incubation at 37° C, the cells were washed twice with PBS and then stained with Annexin V and propidium iodide according to the manufacturer's recommendations. The staining patterns of the Ramos cells were analyzed by flow cytometry using a FACscan Flow Cytometer (Becton Dickinson, San Jose, CA), and data were collected for 10 s-periods. The data were reduced using the Cellquest Pro software (Becton Dickinson). Ramos cells that were positive for (1) Annexin V staining, (2) Annexin V and propiduim iodide double-staining, and (3) the number of unstained live cells, were counted and plotted using KaleidaGraph software (Synergy Software, Reading, PA).

Both rhuMAb 2H7.v16 and Rituximab induced apoptosis of Ramos cells when crosslinked with anti-human Fc and as compared to an irrelevant IgG1 control antibody (Figures 13-15). The apoptotic activity of (rhuMAb 2H7) was slightly lower than that of Rituximab. At 10 nM concentrations of crosslinked rhuMAb 2H7, Rituximab, and control IgG1 antibody, fractions of Annexin V stained cells were 18.5, 16.5, 2.5%, respectively, fractions of doubly labeled cells were 29, 38, and 16%, and numbers of live cells counted per 10 s were 5200, 3100, and 8600.

These *in vitro* data demonstrate that apoptosis is one potential mechanism for *in vivo* B cell depletion. *In vivo* crosslinking of rhuMAb 2H7 or Rituximab bound to cell-surface CD20 may occur through FcγR on the surfaces of immune effector cells.

Example 14

In Vivo Suppression of Tumor Growth

The ability of rhuMAb 2H7.v16 to inhibit the growth of the Raji human B-cells, a lymphoma cell
line (ATCC CCL 86), was evaluated in Balb/c nude (athymic) mice. The Raji cells express CD20 and have
been reported to grow in nude mice, producing metastatic disease; tumor growth is inhibited by Rituxan®
(Clynes et al., *Nature Medicine* **6**, 443-446 (2000)). Fifty-six 8-10 week old, Balb/c nude mice were divided
into 7 groups (A-G) with each group consisting of 8 mice. On day 0, each mouse received a subcutaneous
injection of 5 x 10^6 Raji B-lymphoma cells in the flank. Beginning at day 0, each mouse received either 100
uL of the negative-control solution (PBS; phosphate-buffered saline), Rituxan® or 2H7.v16. Dosage was
dependent on weight and drug delivery was intravenously via the tail vein. Group A mice received PBS.
Groups B-D received Rituxan® at 5.0, mg/kg, 0.5 mg/kg, and 0.05 mg/kg respectively. Groups E-G mice
received 2H7 v.16 at 5.0 mg/kg, 0.5 mg/kg, and 0.05 mg/kg respectively. The injections were repeated every
week for 6 weeks. At weekly intervals during treatment, each mouse was inspected for the presence of
palpable tumors at the site of injection, and the volume of the tumors if present were measured and recorded.
A final inspection was made at week 8 (after a two-week interval of no treatments).

The results of this study showed that both rhuMAb 2H7.v16 and Rituxan® and were effective at
inhibiting subcutaneous Raji-cell tumor growth in nude mice (FIGs. 16-18). Tumor growth was observed in
the PBS control group beginning at 4 weeks. However, no tumor growth was observed in groups treated
with Rituxan® or 2H7.v16 at 5 mg/kg or 0.5 mg/kg for the 8-week duration of the study. In the low-dose
0.05 mg/kg treatment groups, tumors were observed in one animal in the 2H7 group and in one animal in the
Rituxan® group (FIG. 18).

Example 15

Cloning of Cynomolgus monkey CD20 and antibody binding

The CD20 DNA sequence for cynomolgus monkey (Macaca fascicularis) was determined upon the
isolation of cDNA encoding CD20 from a cynomolgus spleen cDNA library. A SUPERSCRIPT™ Plasmid
System for cDNA Synthesis and Plasmid Cloning (Cat#18248-013, Invitrogen, Carlsbad, CA) was used with
slight modifications to construct the library. The cDNA library was ligated into a pRK5E vector using
restriction sites Xho I-and Not I. mRNA was isolated from spleen tissue ((California Regional Research
Primate Center, Davis, CA). Primers to amplify cDNA encoding CD20 were designed based on non-coding
sequences of human CD20. N-terminal region primer 5'-AGTTTTGAGAGCAAAATG-3' and C-terminal
region primer 5'-AAGCTATGAACACTAATG-3' were used to clone by polymerase chain reaction (PCR)
the cDNA encoding cynomolgus monkey CD20. The PCR reaction was carried out using Platinum Taq
DNA Polymerase High Fidelity according to the manufacturers recommendation (Gibco, Rockville, MD).
The PCR product was subcloned into pCR ®2.1-TOPO ® Vector (Invitrogen) and transformed into XL-1 blue
E. coli (Stratagene. La Jolla, CA). Plasmid DNA containing ligated PCR products was isolated from
individual clones and sequenced.

The amino acid sequence for cynomolgus monkey CD20 is shown in Figure 19. Figure 20 shows a
comparison of cynomolgus and human CD20. The cynomolgus monkey CD20 is 97.3% similar to human

5   CD20 with 8 differences. The extracellular domain contains one change at V157A, while the remaining 7
       residues can be found in the cytoplasmic or transmembrane regions.

               Antibodies directed against human CD20 were assayed for the ability to bind and displace FITC-
       conjugated murine 2H7 binding to cynomolgus monkey cells expressing CD20. Twenty milliliters of blood
       were drawn from 2 cynomolgus monkeys (California Regional Research Primate Center, Davis, CA) into
10   sodium heparin and shipped directly to Genentech Inc.. On the same day, the blood samples were pooled and
       diluted 1:1 by the addition of 40 ml of phosphate buffered saline (PBS). 20 ml of diluted blood was layered
       on 4 x 20 ml of Ficoll-Paque ™Plus (Amersham Biosciences, Uppsala, Sweden) in 50 ml conical tubes
       (Cat#352098, Falcon, Franklin Lakes, NJ) and centrifuged at 1300 rpm for 30 minutes R.T. in a Sorval 7
       centrifuge. (Dupont, Newtown, CT). The PBMC layer was isolated and washed in PBS. Red blood cells
15   were lysed in a 0.2% NaCl solution, restored to isotonicity with an equivalent volume of a 1.6% NaCl
       solution, and centrifuged for 10 minutes at 1000 RPM. The PBMC pellet was resuspended in RPMI 1640
       (Gibco, Rockville, MD) containing 5% fetal bovine serum (FBS) and dispensed into a 10 cm tissue culture
       dish for 1 hour at 37° C. The non-adherent B and T cell populations were removed by aspiration,
       centrifuged and counted. A total of 2.4 x 10^7 cells were recovered. The resuspended PBMC were distributed
20   into twenty 12 x 75 mm culture tubes (Cat#352053, Falcon), with each tube containing 1 x 10^6 cells in a
       volume of 0.25 ml. Tubes were divided into four sets of five tubes. To each set was added either media
       (RPMI1640, 5% FBS), titrated amounts of control human IgG_1 antibody, Rituxan®, 2H7.v16, or 2H7.v31,
       The final concentration of each antibody was 30, 10, 3.3 and 1.1 nM. In addition, each tube also received 20
       ul of Fluorescein Isothiocyanate (FITC)-conjugated anti-human CD20 (Cat#555622, BD Biosciences, San
25   Diego, CA). The cells were gently mixed, incubated for 1 hour on ice and then washed twice in cold PBS.
       The cell surface staining was analyzed on a Epic XL-MCL (Coulter, Miami, FL), the geometric means
       derived, plotted (KaleidaGraph™, Synergy Software,, Reading, PA) versus antibody concentrations.

               Data in Figure 21 showed that 2H7 v.16 and 2H7 v.31 competitively displaced FITC-murine 2H7
       binding to cynomolgus monkey cells. Furthermore, Rituxan® also displaced FITC-murine 2H7 binding thus
30   demonstrating that both 2H7 and Rituxan® bind to an overlapping epitope on CD20. In addition, the data
       show that the IC_{50} value for 2H7 v.16, 2H7 v.31 and Rituxan are similar and fall in the 4-6 nM range.

Example 16
Phase I/II study of rhuMAb 2H7 (2H7.v16) in moderate to severe rheumatoid arthritis

35

Protocol Synopsis
A randomized, placebo-controlled, multicenter, blinded phase I/II study of the safety of escalating doses of
PRO70769 (rhuMAb 2H7) in subjects with moderate to severe rheumatoid arthritis receiving stable doses of
concomitant methotrexate.

40

Objectives
The primary objective of this study is to evaluate the safety and tolerability of escalating intravenous (IV)
doses of PRO70769 (rhuMAb 2H7) in subjects with moderate to sever rheumatoid arthritis (RA).

Study Design

This is a randomized, placebo-controlled, multicenter, blinded Phase I/II, investigator- and subject-blinded study of the safety of escalating doses of PRO70769 in combination with MTX in subjects with moderate to sever RA. The study consists of a dose escalation phase and a second phase with enrollment of a larger number of subjects. The Sponsor will remain unblended to treatment assignment.

Subjects with moderate to severe RA who have failed one to five disease-modifying antirheumatic drugs or biologics who currently have unsatisfactory clinical responses to treatment with MTX will be enrolled.

Subjects will be required to receive MTX in the range of 10-25 mg weekly for at least 12 weeks prior to study entry and to be on a stable dose for at least 4 weeks before receiving their initial dose of study drug (PRO70769 or placebo). Subjects may also receive stable doses of oral corticosteroids (up to 10 mg daily or prednisone equivalent) and stable doses of nonsteroidal anti-inflammatory drugs (NSAIDs). Subjects will receive two IV infusions of PRO70769 or placebo equivalent at the indicated dose on Days 1 and 15 according to the following dose escalation plan (see Figure 22).

Dose escalation will occur according to specific criteria (see Dose Escalation Rules) and after review of safety data by an internal safety data review committee and assessment of acute toxicity 72 hours following the second infusion in the last subject treated in each cohort. After the dose escalation phase, 40 additional subjects (32 active and 8 placebo) will be randomized to each of the following dose levels: 2x50 mg, 2x200 mg, 2x500 mg, and 2x1000 mg, if the dose levels have been demonstrated to be tolerable during the dose escalation phase. Approximately 205 subjects will be enrolled in the study.

B-cell counts will be obtained and recorded (for study assessments, see Section 4.5 and Appendix A-1). B-cell counts will be evaluated using flow cytometry in a 48-week follow-up period beyond the 6-month efficacy evaluation. B-cell depletion will not be considered a dose-limiting toxicity (DLC), but rather the expected pharmacodynamic outcome of PRO70769 treatment.

In an optional substudy, blood for serum and RNA analyses, as well as urine samples will be obtained from subjects at various timepoints (see Section 3.3.3). These samples may be used to identify biomarkers that may be predictive of response to PRO70769 treatment in subjects with moderate to severe RA.

Outcome Measures

The primary outcome measure for this study is the safety and tolerability of PRO70769 in subjects with moderate to severe RA.

Study Treatment

Cohorts of subjects will receive two IV infusions of PRO70769 or placebo equivalent at the indicated dose on Days 1 and 15 according to the following escalation plan:

- 10 mg PRO70769 or placebo equivalent: 4 subjects active drug, 1 control
- 50 mg PRO70769 or placebo equivalent: 8 subjects active drug, 2 control
- 200 mg PRO70769 or placebo equivalent: 8 subjects active drug, 2 control
- 500 mg PRO70769 or placebo equivalent: 8 subjects active drug, 2 control
- 1000 mg PRO70769 or placebo equivalent: 8 subjects active drug, 2 control

5   **Efficacy**

The efficacy of PRO70769 will be measured by ACR responses. The percentage of subjects who achieve an ACR20, ACR50, and ACR70 response will be summarized by treatment group and 95% confidence intervals will be generated for each group. The components of these response and their change from baseline will be summarized by treatment and visit.

10

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Conclusion
</div>

The data above demonstrated the success in producing humanized CD20 binding antibodies, in particular humanized 2H7 antibody variants, that maintained and even enhanced their biological properties. The humanized 2H7 antibodies of the invention bound to CD20 at affinities similar to the murine donor and

15   chimeric 2H7 antibodies and were effective at B cell killing in a primate, leading to B cell depletion. Certain variants showed enhanced ADCC over a chimeric anti-CD20 antibody currently used to treat NHL, favoring the use of lower doses of the therapeutic antibody in patients. Additional, whereas it may be necessary for a chimeric antibody that has murine FR residues to be administered at a dose effective to achieve complete B cell depletion to obviate an antibody response against it, the present humanized antibodies can be

20   administered at dosages that achieve partial or complete B cell depletion, and for different durations of time, as desired for the particular disease and patient. In addition, these antibodies demonstrated stability in solution. These properties of the humanized 2H7 antibodies make them ideal for use as immunotherapeutic agent in the treatment of CD20 positive cancers and autoimmune diseases; these antibodies are not expected to be immunogenic or will at least be less immunogenic than fully murine or chimeric anti-CD20 antibodies

25   in human patients.

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References
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References cited within this application, including patents, published applications and other publications, are hereby incorporated by reference.

The practice of the present invention will employ, unless otherwise indicated, conventional

30   techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See *e.g.*, Molecular Cloning: A Laboratory Manual, (J. Sambrook *et al.*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); Current Protocols in Molecular Biology (F. Ausubel *et al.*, eds., 1987 updated); Essential Molecular Biology (T. Brown ed., IRL Press 1991); Gene Expression Technology (Goeddel ed., Academic Press 1991); Methods for Cloning and Analysis of

35   Eukaryotic Genes (A. Bothwell *et al.* eds., Bartlett Publ. 1990); Gene Transfer and Expression (M. Kriegler, Stockton Press 1990); Recombinant DNA Methodology II (R. Wu *et al.* eds., Academic Press 1995); PCR: A Practical Approach (M. McPherson *et al.*, IRL Press at Oxford University Press 1991); Oligonucleotide Synthesis (M. Gait ed., 1984); Cell Culture for Biochemists (R. Adams ed., Elsevier Science Publishers 1990); Gene Transfer Vectors for Mammalian Cells (J. Miller & M. Calos eds., 1987); Mammalian Cell

40   Biotechnology (M. Butler ed., 1991); Animal Cell Culture (J. Pollard *et al.* eds., Humana Press 1990); Culture of Animal Cells, 2nd Ed. (R. Freshney *et al.* eds., Alan R. Liss 1987); Flow Cytometry and Sorting (M. Melamed *et al.* eds., Wiley-Liss 1990); the series Methods in Enzymology (Academic Press, Inc.);Wirth M. and Hauser H. (1993); Immunochemistry in Practice, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, MA, 1996; Techniques in Immunocytochemistry, (G. Bullock & P. Petrusz eds.,

5   Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); Current Protocols in Immunology (J. Coligan et al. eds. 1991); Immunoassay (E. P. Diamandis & T.K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; Ed Harlow and David Lane, Antibodies A laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988; Antibody
10   Engineering, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series Annual Review of Immunology; the series Advances in Immunology.

5   WHAT IS CLAIMED IS:

        1.   A humanized antibody that binds human CD20, or an antigen-binding fragment thereof, wherein
the antibody is effective to deplete primate B cells in vivo, the antibody comprising in the H chain Variable
region (V$_H$) at least a CDR3 sequence of SEQ ID NO. 12 from an anti-human CD20 antibody and
10   substantially the human consensus framework (FR) residues of human heavy chain subgroup III (V$_H$III).

        2.   The antibody of claim 1, further comprising the H chain CDR1 sequence of SEQ ID NO. 10 and
CDR2 sequence of SEQ ID NO. 11.

        3.   The antibody of claim 2, further comprising the L chain CDR1 sequence of SEQ ID NO. 4, CDR2
sequence of SEQ ID NO. 5, CDR3 sequence of SEQ ID NO. 6 and substantially the human consensus
15   framework (FR) residues of human light chain κ subgroup I (VκI).

        4.   The antibody of the preceding claims, comprising the V$_H$ sequence of SEQ ID NO.8 (v16, as shown
in FIG. 1B).

        5.   The antibody of claim 4, further comprising the V$_L$ sequence of SEQ ID NO.2 (v16, as shown in
FIG. 1A).

20   6.   The antibody of claim 3, wherein the V$_H$ region is joined to a human IgG chain constant region.

        7.   The antibody of claim 6, wherein the human IgG is IgG1 or IgG3.

        8.   The antibody of claim 1, wherein the antibody is 2H7.v16 , having the light and heavy chain amino
acid sequence of SEQ ID NO. 21 and 22, respectively, [as shown in FIG. 6 and FIG. 7]

        9.   The antibody of claim 1, wherein the antibody is 2H7.v31 having the light and heavy chain amino
25   acid sequence of SEQ ID NO. 2 and 23, respectively, [as shown in FIG. 6 and FIG. 8].

        10. The antibody of claim 5, but with the amino acid substitutions of D56A and N100A in the H chain
and S92A in the L chain. [v.96]

        11. The antibody of any of the preceding claims, further comprising at least one amino acid substitution
in the Fc region that improves ADCC and/or CDC activity.

30   12. The antibody of claim 11, wherein the amino acid substitutions are S298A/E333A/K334A.

        13. The antibody of claim 12, wherein the antibody is 2H7.v31 having the heavy chain amino acid
sequence of SEQ ID NO. 23 [as shown in FIG. 8].

        14. The antibody of any of claims 1-10, further comprising at least one amino acid substitution in the
Fc region that decreases CDC activity.

35   15. The antibody of any of claim 14, comprising at least the substitution K322A.

        16. The antibody of claim 1-10 wherein the antibody is 2H7.v114 or 2H7.v115 having at least 10-fold
improved ADCC activity as compared to Rituxan.

        17. The antibody of claim 1 wherein the primate B cells are from human and Cynomolgus monkey.

        18. The antibody of any of the preceding claims conjugated to a cytotoxic agent.

40   19. The antibody of claim 18 wherein the cytotoxic agent is a radioactive isotope or a toxin.

        20. The antibody of any of the preceding claims, which antibody is produced in CHO cells.

        21. An isolated nucleic acid that encodes the antibody of any one of the preceding claims.

        22. An expression vector encoding the antibody of any of the preceding claims.

        23. A host cell comprising a nucleic acid of claim 21.

45   24. The host cell of claim 23 that produces the antibody of any one of preceding claims.

25. The host cell of claim 24 which is a CHO cell.

26. A method of producing the antibody of any one of the preceding claims, comprising culturing the cell that produces the antibody of claim 24 and recovering the antibody from the cell culture.

27. A composition comprising the antibody of claim 1 and a carrier.

28. The composition of claim 27 wherein the antibody is 2H7.v16 and the carrier is a pharmaceutically acceptable carrier.

29. An article of manufacture comprising a container and a composition contained therein, wherein the composition comprises an antibody of any of the preceding claims.

30. The article of manufacture of claim 29, further comprising a package insert indicating that the composition can be used to treat non-Hodgkin's lymphoma.

31. A method of inducing apoptosis in B cells in vivo, comprising contacting B cells with the antibody of any of the preceding claims, thereby killing the B cells.

32. A method of treating a CD20 positive cancer, comprising administering to a patient suffering from the cancer, a therapeutically effective amount of the humanized CD20 binding antibody of any of the preceding claims.

33. The method of claim 32 wherein the CD20 positive cancer is a B cell lymphoma or leukemia.

34. The method of claim 33 wherein CD20 positive cancer is non-Hodgkin's lymphoma (NHL) or lymphocyte predominant Hodgkin's disease (LPHD).

35. The method of claim 32 wherein the cancer is chronic lymphocytic leukemia or SLL.

36. The method of claims 34 or 35 wherein the antibody is selected from the group consisting of 2H7.v16, v31.v96, v114, v115 having the respective amino acid sequences as shown in the figures and tables.

37. The method of claim 34 or 35 wherein the antibody is 2H7.v16 having the light and heavy chain amino acid sequence of SEQ ID NO. 21 and 22, respectively, as shown in FIG. 6 and FIG. 7.

38. The method of claim 33, wherein the antibody is administered at a dosage range of about 275-375mg/m^2.

39. The method of claim 32, further comprising administering to the patient at least one chemotherapeutic agent.

40. The method of claim 39, wherein the cancer is non-Hodgkin's lymphoma (NHL) and the chemotherapeutic agent is selected from the group consisting of doxorubicin, cyclophosphamide, vincristine and prednisolone.

41. A method of treating an autoimmune disease, comprising administering to a patient suffering from the autoimmune disease, a therapeutically effective amount of the humanized CD20 binding antibody of any one of the preceding claims.

42. The method of claim 41, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome, Sjorgen's syndrome and glomerulonephritis.

43. The method of claim 42, wherein the autoimmune disease is rheumatoid arthritis.

44. The method of claim 43, further comprising administering to the patient a second therapeutic agent.

45. The method of claim 44, wherein the second therapeutic agent is an immunosuppressive agent.

46. The method of claim 45, wherein the immunosuppressive agent is methotrexate.

47. A method of treating an autoimmune disease selected from the group consisting of Dermatomyositis, Wegner's granulomatosis, ANCA (included under vasculitis), Aplastic anemia, Autoimmune hemolytic anemia (AIHA), factor VIII deficiency, hemophilia A, Autoimmune neutropenia, Castleman's syndrome, Goodpasture's syndrome, solid organ transplant rejection, graft versus host disease (GVHD), IgM mediated, thrombotic thrombocytopenic purpura (TTP), Hashimoto's Thyroiditis, autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs. NSIP, Guillain-Barre Syndrome, large vessel vasculitis, giant cell (Takayasu's) arteritis, medium vessel vasculitis, Kawasaki's Disease, and polyarteritis nodosa, comprising administering to a patient suffering from the disease, a therapeutically effective amount of a CD20 binding antibody or functional fragment thereof.

48. The method of claim 47 wherein the CD20 binding antibody is Rituxan®.

49. An isolated nucleic acid comprising the nucleotide sequence of SEQ ID NO.: _ of the Cynomolgus monkey CD20 (shown in FIG. 19), or a degenerate variant of this sequence.

50. An isolated nucleic acid comprising a sequence that encodes a polypeptide with the amino acid sequence of SEQ ID NO. _ (shown FIG. 20), or SEQ ID NO. _ (FIG. 20) with conservative amino acid substitutions.

51. A vector comprising the nucleic acid of claim 50.

52. The vector of claim 51 which is an expression vector comprising the nucleic acid of claim 49 operably lined to an expression control sequence.

53. A host cell comprising the nucleic acid of claim 50.

54. An isolated polypeptide comprising the amino acid sequence [SEQ ID NO. _ ; FIG. 20] of the Cynomolgus monkey CD20.

55. A liquid formulation comprising a humanized 2H7 antibody at 20mg/mL, 10mM histidine sulfate at pH5.8, 60mg/ml sucrose, 0.2 mg/ml polysorbate 20.

FIG. 1A

Sequence alignment of variable light-chain domain

```
                  |———————FR1 ————|        CDR1          |————|
                      10        20            30              40
2H7          QIVLSQSPAILSASPGEKVTMTC  [RASSSVS-YMH]  WYQQKP
             *  ***    **      *  **  *
hu2H7.v16    DIQMTQSPSSLSASVGDRVTITC  [RASSSVS-YMH]  WYQQKP
                                                      *  *  *  **
hum κI       DIQMTQSPSSLSASVGDRVTITC  [RASQSISNYLA]  WYQQKP
```

```
              FR2————|    CDR2   |——————————FR3————————
                      50              60        70          80
2H7          GSSPKPWIY  [APSNLAS]  GVPARFSGSGSGTSYSLTISRVEA
             **    *                   *        ***    ****
hu2H7.v16    GKAPKPLIY  [APSNLAS]  GVPSRFSGSGSGTDFTLTISSLQP
                *           *  *  *
hum κI       GKAPKLLIY  [AASSLES]  GVPSRFSGSGSGTDFTLTISSLQP
```

```
              ———————|   CDR3     |——FR4—|
                     90              100
2H7          EDAATYYC  [QQWSFNPPT]  FGAGTKLELKR
                *                      *   *  *
hu2H7.v16    EDFATYYC  [QQWSFNPPT]  FGQGTKVEIKR
                         ****  *
hum κI       EDFATYYC  [QQYNSLPWT]  FGQGTKVEIKR
```

FIG. 1B

Sequence alignment of variable heavy-chain domain

```
              |————————FR1 ————————|        CDR1            |————|
                        10            20              30           40
2H7        QAYLQQSGAELVRPGASVKMSCKAS   [GYTFTSYNMH]   WVKQT
           *** ** ** * * *** *                        * *
hu2H7.v16  EVQLVESGGGLVQPGGSLRLSCAAS   [GYTFTSYNMH]   WVRQA
                                          *  *  *  *
hum III    EVQLVESGGGLVQPGGSLRLSCAAS   [GFTFSSYAMS]   WVRQA


            —FR2—|   50   a      CDR2          |————FR3 —|
                                  60              70        80
2H7        PRQGLEWIG  [AIYPGNGDTSYNQKFKG]   KATLTVDKSSSTAYM
           **      *                        ** **    ** * *
hu2H7.v16  PGKGLEWVG  [AIYPGNGDTSYNQKFKG]   RFTISVDKSKNTLYL
                  *    * .**** * * ****          * *
hum III    PGKGLEWVA  [VISGDGGSTYYADSVKG]   RFTISRDNSKNTLYL


            |—————————————————|        CDR3           |—FR4—|
               abc       90            100abcde           110
2H7        QLSSLTSEDSAVYFCAR   [VVYYSNSYWYFDV]  WGTGTTVTVSS
           **   **  *   *                        *   *
hu2H7.v16  QMNSLRAEDTAVYYCAR   [VVYYSNSYWYFDV]  WGQGTLVTVSS
                \                ****** *** *
hum III    QMNSLRAEDTAVYYCAR   [GRVGYSLY---DY]  WGQGTLVTVSS
```

FIG. 2-1
PVX4

```
  1  GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
     CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTTCTTCT TCTCAGCTTA

101  GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
     CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC TGGTTGTCGC CAACTAACTA GTCCATCTCC

201  GGGCGCCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
     CCCGCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

301  AAAAGTTAAT CTTTTCAACA GCTGTCATPA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTTTTTATT TTTTAAGTA  GAATTCGAGC
     TTTTCAATTA GAAAAGTTGT CGACAGTGTT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT CTTAAGCTCG

401  TCGGTACCCG GGGATCCTCT AGAGGTTGAG GTGATTTTAT GAAAAAGAAT ATCGCATTTC TTCTTGCATC TATGTTCGTT CTACAAACGC
     AGCCATGGGC CCCTAGGAGA TCTCCAACTC CACTAAAATA CTTTTTCTTA TAGCGTAAAG AAGAACGTAG ATACAAGCAA GATGTTTGCG
  1                                                               M  K  K  N  I  A  F  L  L  A  S  M  F  V  L  Q  T  N  A

501  GTACGCTGAT ATCCAGATGA CCCAGTCCCC GAGCTCCCTG TCCGCCTCTG TGGGCGATAG GGTCACCATC ACCTGCAGAG CGTGTCGACT
     CATGCGACTA TAGGTCTACT GGGTCAGGGG CTCGAGGGAC AGGCGGAGAC ACCCGCTATC CCAGTGGTAG TGGACGTCTC GCACAGCTGA
 22  Y  A  D  I  Q  M  T  Q  S  P  S  S  L  S  A  S  V  G  D  R  V  T  I  T  C  R  A  S  Q  S  V  S  T

601  AGCTCCTATA CTGGTATCAA CAGAAACCAG GGAAAGCTCC ATTTACTATG CGAGTCTGGA GCTCAGACCT CAGGGAAGAG GTCCCTTCTC
     TCGAGGATAT GACCATAGTT GTCTTTGGTC CCTTTCGAGG TAAATGATAC GCTCAGACCT CGAGTCTGGA GTCCCTTCTC CAGGGAAGAG
 55  S  S  Y  M  H  W  Y  Q  Q  K  P  G  K  A  P  K  L  L  I  Y  Y  A  S  N  L  E  S  G  V  P  S  R

701  GCTTCTCTGG ATCCGGGTTCT GGGACGGATT TCACTCTGAC CATCAGCAGT CTGCAGCCAG AAGACTTCGC TGTCAACACT CTTGGGTAT
     CGAAGAGACC TAGGCCCTAA CCCTGCCTAA AGTGAGACTG GTAGTCGTCA GACGTCGGTC TTCTGAAGCG ACAGTTGTGA GAACCCCATA
 89  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  P  E  D  F  A  T  Y  Y  C  Q  H  S  W  G  I

801  TCCGCGCCACA CGAACTGTGG CTGCACCATC TGTCTTCATC TTCCCGCCAT CTGATGAGCA GTTGAAATCT
     AGGCGCGGTGT GCTTGACACC GACGTGGTAG ACAGAAGTAG AAGGGCGGTA GACTACTCGT CAACTTTAGA
122  P  R  T  F  G  Q  G  T  K  V  E  I  K  R  T  V  A  A  P  S  V  F  I  F  P  P  S  D  E  Q  L  K  S

901  GGAACTGCTT CTGTTGTGTG CCTGCTGAAT AACTTCTATC CCAGAGAGGC CAAAGTACAG TGGAAGGTGG ATAACGCCCT AACTCCCAGG
     CCTTGACGAA GACAACACAC GGACGACTTA TTGAAGATAG GGTCTCTCCG GTTTCATGTC ACCTTCCACC TATTGCGGGA TTGAGGGTCC
155  G  T  A  S  V  V  C  L  L  N  N  F  Y  P  R  E  A  K  V  Q  W  K  V  D  N  A  L  Q  S  G  N  S  Q  E

1001 AGAGTGTCAC AGAGCAGGAC AGCAAGGACA GCACCTACAG CCTCAGCAGC ACCCTGACGC TGAGCAAAGC AGACTACGAG AAACACAAAG TCTACGCCTG
     TCTCACAGTG TCTCGTCCTG TCGTTCCTGT CGTGGATGTC GGAGTCGTCG TGGGACTGCG ACTCGTTTCG TCTGATGCTC TTTGTGTTTC AGATGCGGAC
189  S  V  T  E  Q  D  S  K  D  S  T  Y  S  L  S  S  T  L  T  L  S  K  A  D  Y  E  K  H  K  V  Y  A  C
```

FIG. 2-2
PVX4

```
1101  CGAAGTCACC CATCAGGGCC TGAGCTCGCC CGTCACAAAG AGCTTCAACA GGGGAGAGTG TTAAGCTGAT CCTCCTACGCC TGGCCCTAGT
      GCTTCAGTGG GTAGTCCCG  ACTCGAGCGG GCAGTGTTTC TCGAAGTTGT CCCCTCTCAC AATTCGACTA GGAGATGCGG CCTGGGATCA
       E  V  T   H  Q  G  L   S  S  P   V  T  K   S  F  N  R   G  E  C   Q
222

1201  ACGCAAGTTC ACGTAAAAAG GGTATCTAGA ATTTATGAA  AAAGAATATC GCATTCTTC  TTGCATCTAT GTTCGTTTTT TCTATTGCTA
      TGCGTTCAAG TGCATTTTTC CCATAGATCT TAAAATACTT TTTCTTATAG CGTAAGAAG  AACGTAGATA CAAGCAAAAA AGATAACGAT
                                        M  K  N  I   A  F  L  L   A  S  M   F  V  F   S  I  A  T
  1

1301  CAAACGCGTA CGCTGAGGTT CAGCTGGTGG AGTCTGGCGG TGGCCTGGTG CAGCCAGGGG GCTCACTCCG TTTGTCCTGT GCTACACCTT
      GTTTGCGCAT GCGACTCCAA GTCGACCACC TCAGACCGCC ACCGGACCAC GTCGGTCCCC CGAGTGAGGC AAACAGGACA CGATGTGGAA
       N  A  Y   A  E  V   Q  L  V  E   S  G  G   G  L  V   Q  P  G  G   S  L  R   L  S  C   A  A  S  G   Y  T  F
 20

1401  CACCGAATAT ATCATCCACT GGGTCCGTCA GGCCCCGGGT AAGGGCCTGG AATGGGTTGC ATCGATTAAT CCTGACTACG CTATAACCAG
      GTGGCTTATA TAGTAGGTGA CCCAGGCAGT CCGGGGCCCA TTCCCGGACC TTACCCAACG TAGCTAATTA GGACTGATGC GATATTGGTC
       T  E  Y   I  I  H  W   V  R  Q   A  P  G   K  G  L  E   W  V  A   S  I  N   P  D  Y  D   I  T  N   Y  N  Q
 53

1501  CGCTTCAAGG TATAAGTCGC GCCGTTTCAC ATACCTGCAG ATGAACAGCC TGCGTGCTGA GGACACTGCC GTCTATTATT
      GCGAAGTTCC ATATTCAGCG CGGCAAAGTG TATGGACGTC TACTTGTCGG ACGCACGACT CCTGTGACGG CAGATAATAA
       R  F  K  G   R  F  T   I  S  R   D  D  S  K   N  T  L   Y  L  Q   M  N  S  L   R  A  E   D  T  A   V  Y  Y  C
 86

1601  GTGCTCGATG GATCAGCGAT TTCTTCGACT ACTGGGGTCA AGGAACCCTG GTCACCGTCT CCTCGGCCTC CCATCGGTCT TCCCCCTGGC
      CACGAGCTAC CTAGTCGCTA AAGAAGCTGA TGACCCCAGT TCCTTGGGAC CAGTGGCAGA GGAGCCGGAG GGTAGCCAGA AGGGGGACCG
       A  R  W   I  S  D   F  F  D  Y   W  G  Q   G  T  L   V  T  V  S   S  A  S   T  K  G   P  S  V  F   P  L  A
120

1701  ACCCTCCTCC AAGAGCACCT CTGGGGGCAC AGCGGCCCTG GGCTGCCTGG TCAAGGACTA CTTCCCCGAA CCGGTGACGG CTCAGGCGGCC
      TGGGAGGAGG TTCTCGTGGA GACCCCCGTG TCGCCGGGAC CCGACGGACC AGTTCCTGAT GAAGGGGCTT GGCCACTGCC GAGTCCGCCG
       P  S  S   K  S  T  S   G  G  T   A  A  L   G  C  L  V   K  D  Y   F  P  E   P  V  T  V   S  W  N   S  G  A
153

1801  CTGACCAGCG GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC AGCAGCGTGG TGACCGTGCC CTCCAGCAGC TTGGGCACCC
      GACTGGTCGC CGCACGTGTG GAAGGGCCGA CAGGATGTCA GGAGTCCTGA GATGAGGGAG TCGTCGCACC ACTGGCACGG GAGGTCGTCG AACCCGTGGG
       L  T  S  G   V  H  T   F  P  A   V  L  Q  S   S  G  L   Y  S  L   S  S  V  V   T  V  P   S  S  S   L  G  T  Q
186

1901  AGACCTACAT CTGCAACGTG AATCACAAGC CCAGCAACAC CAAGGTGGAC AAGAAAGTTG AGCCCAAATC TTGTGACAAA ACTCACACAT GACCACCGCA
      TCTGGATGTA GACGTTGCAC TTAGTGTTCG GGTCGTTGTG GTTCCACCTG TTCTTTCAAC TCGGGTTTAG AACACTGTTT TGAGTGTGTA CTGGTGGCGT
       T  Y  I   C  N  V   N  H  K  P   S  N  T   K  V  D   K  K  V  E   P  K  S   C  D  K   T  H  T  Q
220

2001  TGCACCAGTA TCGTCCATTC CGACAGCATC GCCAGTCACT ATGGCGTGCT GCTAGCGCCG CCCTATACCT GTGTCTGCCTC CCCGCGGTTGC GTCGCGGTGC
      ACGTGGTCAT AGCAGGTAAG GCTGTCGTAG CGGTCAGTGA TACCGCACGA CGATCGCGGC GGGATATGGA ACAGACGGAG GGGGCGCCAACG CAGCGCCACG

2101  ATGGAGCCGG GCCACCTGA  AGCCGGCGCG CCTGAATGGA TCAATTCTTG CGGAGAACTG
      TACCTCGGCC CGGTGGAGCT TCGGCCGCGC GGACTTACCT AGTTAAGAAC GCCTCTTGAC

2201  TGAATGCGCA AACCAACCCT TGGCAGAACA TATCCATCGC GTCCGCCATC TCCAGCAGCC AGGCGTTGGGT CCTGGCCACG AGCGTTGGGT CCTGGCCACG
      ACTTACGCGT TTGGTTGGGA ACCGTCTTGT ATAGGTAGCG CAGGCGGTAG AGGTCGTCGG TCCGCAACCCCA GGACCGGTGC TCGCAACCCCA GGACCGGTGC
```

FIG. 2-3
PVX4

```
2301  GGTGCGCCATG  ATCGTGTCTCC  TGTCGTTGAG  GACCCGGCTA  GGCTGGCGGG  GTTGCCTTAC  TGGTTAGCAG  AATGAATCAC  CGATACGGCGA  GCGAACGTGA
      CCACGCGTAC  TAGCACGAGG  ACAGCAACTC  CTGGGCGGAT  CCGACCGCCC  CAACGGAATG  ACCAATCGTC  TTACTTAGTG  GCTATGCGCT  CGCTTGCACT

2401  AGCCGACTGCT  GCTGCAAAAC  GTCTGCGACC  TGAGCAACAA  CATGAATGGT  CTTCGGTTTC  CGTGTTTCGT  AAAGTCTGGA  AACGCGGAAG  TCAGCGCCCT
      TCGGCTGACGA  CGACGTTTTG  CAGACGCTGG  ACTCGTTGTT  GTACTTACCA  GAAGCCAAAG  GCACAAAGCA  TTTCAGACCT  TTGCGCCTTC  AGTCGCGGGA

2501  GCACCATTAT  GTTCCGGATC  TGCATCGCAG  GATGCTGCTG  GCTACCCTGT  GGAACACCTA  CATCTGTATT  AACGAAGCGC  TGGCCATTGAC  CCTGAGTGAT
      CGTGGTAATA  CAAGGCCTAG  ACGTAGCGTC  CTACGACGAC  CGATGGGACA  CCTTGTGGAT  GTAGACATAA  TTGCTTCGCG  ACCGGTAACTG  GGACTCACTA

2601  TTTTCTCTGG  TCCCGCCGCA  TCCATACCGC  CAGTTGTTTA  CCCTCACAAC  GTTCCAGTAA  CCGGGCATGT  TCATCATCAG  TAACCCGTAT  CGTGAGCATC
      AAAAGAGACC  AGGGCGGCGT  AGGTATGGCG  GTCAACAAAT  GGGAGTGTTG  CAAGGTCATT  GGCCCGTACA  AGTAGTAGTC  ATTGGGCATA  GCACTCGTAG

2701  CTCTCTCGTT  TCATCGGTAT  CATTACCCCC  ATGAACAGAA  ATTCCCCCT  ACACGGAGGC  ATCAAGTGAC  CAAACAGGAA  AAAACCGCCC  TTAACATGGC
      GAGAGAGCAA  AGTAGCCATA  GTAATGGGGG  TACTTGTCTT  TAAGGGGGAA  TGTGCCTCCG  TAGTTCACTG  GTTTGTCCTT  TTTTGGCGGG  AATTGTACCG

2801  CCGCTTTATC  AGAAGCCAGA  CATTAACGCT  TCTGGAGAAA  CTCAACGAGC  TGGACGCGGA  GAACAGGCGA  GACATCTGTG  AATCGCTTCA  CGACCACGCT
      GGCGAAATAG  TCTTCGGTCT  GTAATTGCGA  AGACCTCTT  GAGTTGCTCG  ACCTGCGCCT  CTTGTCCGT  CTGTAGACAC  TTAGCGAAGT  GCTGGTGCGA

2901  GATGAGCTTT  ACCGCAGCAT  TAAAACGTTAA  TATTTTGTTA  AAATTCGCGT  TTAAATCAGC  TCATTTTTTA  ACCAATAGGC  GAACGTGGAC
      CTACTCGAAA  TGGCGTCGTA  ATTTGCAATT  ATAAAACAAT  TTTAAGCGCA  AATTTAGTCG  AGTAAAAAAT  TGGTTATCCG  CTTGCACCTG

3001  CGAAATCGGC  AAAATCCCTT  ATAAATCAAA  AGAATAGACC  GAGATAGGGT  TGAGTGTTGT  TCCAGTTTGG  AACAAGAGTC  CACTATTAAA  GAACGTGGAC
      GCTTTAGCCG  TTTTAGGGAA  TATTTAGTTT  TCTTATCTGG  CTCTATCCCA  ACTCACAACA  AGGTCAAACC  TTGTTCTCAG  GTGATAATTT  CTTGCACTG

3101  TCCAACGTCA  AAGGGGCGAAA  AACCCTCTAT  CAGGGCTATG  GCCCACTACG  TGAACCATCA  CCCTAATCAA  GTTTTTTGGG  GTCGGAGGTGC  CGTAAAGCAC
      AGGTTGCAGT  TTCCCGCTTT  TTGGCAGATA  GTCCCGATAC  GGGGTGATGC  ACTTGGTAGT  GGGATTAGTT  CAAAAAACCC  CAGCTCCACG  GCATTCGTG

3201  TAAAATCGGAA  CCCTAAAGGG  TTAGAGCTTG  ACGGGGAAAG  CCGGCGAACG  TGGCCGAGAA  GGAAGGGAAG  AAAGCGAAAG  'GAGCGGGCGC
      ATTTAGCCTT  GGGGATTCCC  TCGGGGGCTA  AATCTCGAAC  TGCCCCGTTGC  GGCCGCTTGC  ACCGCTCTTT  CCTTCCCTTC  CTCGCCCGCG

3301  TAGGGCGCTG  GCAAGTGTAG  CGGTCACGCT  GCGCGTAACC  ACCACACCCG  ATGCAGCTCC  TGCGCCGCTA  CAGGGGCGCGT  CCGCATCCTG  CCTCGCGCGT
      ATCCCGCGAC  CGTTCACATC  GCCAGTGCGA  CGCGCATTGG  TGGTGTGGGC  TACGTCGAGG  ACGCGGCGGAT  GTCCCGCGCA  GGCGTAGGAC  GGAGCGCGCA

3401  TTCGGTGATG  ACGGTGAAAA  ATACCGCACA  CCTCTGACAC  CGGAGACGGT  CACAGCTGT  CGTAAGGCGG  ATGCCGGGAG  CAGACAAGCC  CGTCAGGGCG
      AAGCCACTAC  TGCCACTTTT  TATGGCGTGT  GGAGACTGTG  GCCTCTGCCA  GTGTCGAACA  GCATTCCGCC  TACGGCCCTC  GTCTGTTCGG  GCAGTCCCGC

3501  CGTCAGCGGG  TGTTGGCGGG  TGTCTGGGGG  CCAGCCATGAC  CCAGTCACGT  AGCGATAGCG  GAGTGTATAC  TGGCTTAACT  ATGCGGCATC  AGAGCAGATT
      GCAGTCGCCC  ACAACCGCCC  ACAGACCCCC  GGTCGGTACTG  GTCAGTGCA  TCGCTATCGC  CTCACATATG  ACCGAATTGA  TACGCCGTAG  TCTCGTCTAA

3601  GTACTGAGAG  TGCACCATAT  GCGGTGTGAA  ATACCGCACA  GATGCGTAAG  GAGAAAATAC  CGCATCAGGC  GCTCTTCCGC  TTCCTCGCTC  ACTGACTCGC
      CATGACTCTC  ACGTGGTATA  CGCCACACTT  TATGGCGTGT  CTACGCATTC  CTCTTTTATG  GCGTAGTCCG  CGAGAAGGCG  AAGGAGCGAG  TGACTGAGCG

3701  TGCGCTCGGT  CGTTCGGCTG  CGGCGAGCGG  TATCAGCTCA  CTCAAAGGCG  GTAATACGGT  TATCCACAGA  ATCAGGGGAT  AACGCAGGAA  AGAACATGTG
      ACGCGAGCCA  GCAAGCCGAC  GCCGCTCGCC  ATAGTCGAGT  GAGTTTCCGC  CATTATGCCA  ATAGGTGTCT  TAGTCCCCTA  TTGCGTCCTT  TCTTGTACAC
```

FIG. 2-4
PVX4

```
3801  AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACACA AAATCGACGC
      TCGTTTTCCG GTCGTTTTCC GGTCCTTGGC ATTTTTCCGG CGCAACGACC GCAAAAAGGT ATCCGAGGCG GGGGGACTGC TCGTAGTGTT TTTAGCTGCG

3901  TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGGCGCTCTC TGTTCCGACC CTGCCGCTTA
      AGTTCAGTCT CCACCGCTTT GGGCTGTCCT GATATTTCTA TGGTCCGCAA AGGGGGACCT TCGAGGGAGC ACGGCGAGAG ACAAGGCTGG GACGGCGAAT

4001  CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAT AGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT
      GGCCTATGGA CAGGCGGAAA GAGGGAAGCC CTTCGCACCG CGAAAGAGTA TCGAGTGCGA CATCCATAGA GTCAAGCCAC ATCCAGCAAG CGAGGTTCGA

4101  GGGCTGTGTG CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCG GTAACTATCG GACACGACTT ATCGCCACTG
      CCCGACACAC GTGCTTGGGG GGCAAGTCGG GCTGGCGACG CGGAATAGGC CATTGATAGC AGAACTCAGG TTGGGCCATT CTGTGCTGAA TAGCGGTGAC

4201  GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTACTA CGGCTACACTA AGAAGGACAG
      CGTCGTCGGT GACCATTGTC CTAATCGTCT CGCTCCATAC ATCCGCCACG AACTTCACCA CCGGATTGAT GCCGATGTGA TCTTCCTGTC

4301  TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT CTTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGAACGA AAACTCACGT
      ATAAACCATA GACGCGAGAC GACTTCGGTC AATGGAAGCC TTTTTCTCAA CCATCGAGAA GAAACTAGAA AAGATGCCCC AGACTGCGAG TCACCTTGCT TTTGAGTGCA

4401  TGTTTGCAAG CAGCAGATTA TTATCAAGA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTTAAATTAA AAATGAAGTT TTATTAGTTA CTAAAGTATA TATGAGTAAA
      ACAAACGTTC GTCGTCTAAT AATAGTTTT TTTTTCCTAGA GTTCTTCTAG GAAACTAGAA AAATTTAATT TTTACTTCAA AATTTCATAT GATTTCATAT ATACTCATTT

4501  TAAGGGATTT TGGTCATGAG ATTATCAAGA AAAAGGATCT CAAGAAGATC CCTAGATCCT TTTAAATTAA TTCGTTCATC CATAGTTGCC TGACTCCCCG TCGTGTAGAT
      ATTCCCTAAA ACCAGTACTC TAATAGTTTT TTTTTCCTAGA GTTCTTCTAG GGATCTAGGA AAATTTAATT AAGCAGTAG GTATCAACGG ACTGAGGGGC AGCACATCTA

4601  CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC TATTCAGCGC ATCTGTCTAT TTCGTTCATC CATAGTTGCC GCTCCAGATT TATCAGCAAT AAACCAGCCA
      GAACCAGACT GTCAATGGTT ACGAATTAGT CACTCCGTGG ATAGAGTCGC TAGACAGATA AAGCAGTAG GTATCAACGG CGAGGTCTAA ATAGTCGTTA TTTGGTCGGT

4701  AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCTCCAGATT
      TTGATGCTAT GCCCTCCCGA ATGGTAGACC GGGGTCACGA CGTTACTATG GCGCTCTGGG TGCGAGTGGC CGAGGTCTAA ATAGTCGTTA TTTGGTCGGT

4801  GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT CCAGTCTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG AGTAAGTAGT TCGCCAGTTA
      CGGCCTTCCC GGCTCGCGTC TTCACCAGGA GGTCAGAATA GGCGGAGGTA GGTCAGATAA TTAACAACGG CCCTTCGATC TCATTCATCA AGCGGTCAAT

4901  ATAGTTTGCG CAACGTTGTT GCCATTGCTG CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG
      TATCAGTACT GTTGCAACAA CGGTAACGAC GTCCGTAGCA CCACAGTGCG AGCAGCAAAC CATACCGAAG TAAGTCGAGG CCAAGGGTTG CTAGTTCCGC

5001  AGTAGTTTGCG TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT
      TCAATGTACT AGGGGGTACA ACACGTTTTT TCGCCAATCG AGGAAGCCAG GAGGCTAGCA ACAGTCTTCA TTCAACCGGC GTCACATAG TGAGTACCAA

5101  ATGGCAGCAC TGCATAATTC TCTTACTGTC TTGCTCTTGC CCGGCGTCAA TACGGGATAA CACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT CTTCGGGGCG
      TACCGTCGTG ACGTATTAAG AGAATGACAG GGCCGCAGTT ATGCCCGCGT GTGCCCTATT GTATCGTCTT GAAATTTTCA CGAGTAGTAA GAAGCCCCGC

5201  GGCGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA CACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG
      CCGCTGGCTC AACGAGAACG GGCCGCAGTT ATGCCCGCGT GTGCCCTATT GTATCGTCTT GAAATTTTCA CGAGTAGTAA CCTTTTGCAA GAAGCCCCGC
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FIG. 2-5
PVX4

5301 AAAACTCTCA AGGATTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT TTACTTTCAC CAGCGTTTCT
      TTTTGAGAGT TCCTAGAATG GCGACAACTC TAGGTCAAGC TACATTGGGT GAGCACGTGG GTTGACTAGA AATGAAAGTG GTCGCAAAGA

5401 GGGTGAGCAA AAACAGGAAG GCAAAATGCC GAATAAAAGG GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA
      CCCACTCGTT TTTGTCCTTC CGTTTTTACGG CTTATTCCCG CTGTGCCTTT ACAACTTATG AGTATGAGAA GGAAAAAGTT ATAATAACTT

5501 GCATTATCA GGGTTATTGT CTCATGAGCG GATACACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC GAAAAGTGCC
      CGTAAATAGT CCCAATAACA GAGTACTCGC CTATGTATAA ACTTACATAA ATCTTTTTAT TTGTTTATCC CCAAGGCGCG CTTTTTCACGG

5601 ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGGCGTATCAC GAGGCCCTTT CGTCTTCAA
      TGGACTGCAG ATTCTTTGGT AATAATAGTA CTGTAATTGG ATATTTTTAT CCCGCATAGTG CTCCGGGAAA GCAGAAGTT

FIG 3-1

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  1  GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
     CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTTCTTCT TCTCAGCTTA

101  GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAATATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
     CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC TGGTTGTCGC CAACTAACTA GTCCATCTCC

201  GGGCGGCTGTA CGAGGTAAAG CCCGATGCCA GCGATTCCTGA GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
     CCCGCCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

301  AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTTTTTATT TTTGTAACTA GAATTCGAGC
     TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAACATTGAT CTTAAGCTCG

401  TCGGTACCCG GGGATCCTCT AGAGGTTGAG GTGATTTATG AAAAAGAATA TCGCATTTCT TCTTGCATCT ATGTTCGTTT TACAAACGCG
     AGCCATGGGC CCCTAGGAGA TCTCCAACTC CACTAAATAC TTTTTCTTAT AGCGTAAAGA AGAACGTAGA TACAAGCAAA ATGTTTGCGC
  1                                                   M   K   K   N   I   A   F   L   L   A   S   M   F   V   F

501  TACGCTCAGA TAGTACTGTC CCAGTCCCCG GCTATCCTGT CCGCCCTCTCC TGGCGAGAAG GTCACTATGA CCTGCAGAGC GTGAGCTATA
     ATGCGAGTCT ATCATGACAG GGTCAGGGGC CGATAGGACA GGCGGGAGAGG ACCGCTCTTC CAGTGATACT GGACGTCTCG CACTCGATAT
 22   Y   A   Q   I   V   L   S   Q   S   P   A   I   L   S   A   S   P   G   E   K   V   T   M   T   C   R   A   S   S   S   V   S   Y   M

601  TGCATTGGTA TCAACAGAAA CCAGGAAGCT CTCCGAAAACC ATGGATTAC GCTCCATCGA ACCTCGCGTC CTGGAGTCCCT CTTGGATCCGG
     ACGTAACCAT AGTTGTCTTT GGTCCTTCGA GAGGCTTTGG TACCTAAATG CGAGGTAGCT TGGAGCGCAG GACCTCAGGGA GAACCTAGGCC
 56   H   W   Y   Q   Q   K   P   G   S   S   P   K   P   W   I   Y   A   P   S   N   L   A   S   G   V   P   A   R   F   S   ,   G   S   G

701  TTCTGGGACT AGTTACTCTC TGACCATCAG CAGAGTGGAG GCAGAAGACG CCGCAACTTA TTACTGTCAA CAGTGGAGCT TCAATCCGCC CACATTTGGA
     AAGACCCTGA TCAATGAGAG ACTGGTAGTC GTCTCACCTC CGTCTTCTGC GGCGTTGAAT AATGACAGTT GTCACCTCGA AGTTAGGCGG GTGTAAACCT
 89   S   G   T   S   Y   S   L   T   I   S   R   V   E   A   E   D   A   A   T   Y   C   Q   Q   W   S   F   N   P   P   T   F   G

801  GCCGGCCACCA AGCTGGAGCT CAAACGAACT GTGGCTGCAC CATCTGTCTT CATCTTCCCG CCATCTGATG AGCAGTTGAA ATCTGGAACT GCTTCTGTTG
     CGGCCGGTGGT TCGACCTCGA GTTTGCTTGA CACCGACGTG GTAGACAGAA GTAGAAGGGC GGTAGACTAC TCGTCAACTT TAGACCTTGA CGAAGACAAC
122   A   G   T   K   L   E   L   K   R   T   V   A   A   P   S   V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A   S   V   V

901  TGTGCCTGCT GAATAACTTC TATCCCAGAG AGGCCAAAGT ACAGTGGAAG GTGGATAACG CCCTCCAATC GGGTAACTCC CAGGAGAGTG TCACAGAGCA
     ACACGGACGA CTTATTGAAG ATAGGGTCTC TCCGGTTTCA TGTCACCTTC CACCTATTGC GGGAGGTTAG CCCATTGAGG GTCCTCTCAC AGTGTCTCGT
156   C   L   L   N   N   F   Y   P   R   E   A   K   V   Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S   V   T   E   Q

1001 GGACAGCAAG GACAGCACCT ACAGCCTCAG CAGCACCCTG ACGCTGAGCA AAGCAGACTA CGAGAAACAC AAAGTCTACG CCTGCGAAGT CACCCATCAG
     CCTGTCGTTC CTGTCGTGGA TGTCGGAGTC GTCGTGGGAC TGCGACTCGT TTCGTCTGAT GCTCTTTGTG TTTCAGATGC GGACGCTTCA GTGGGTAGTC
189   D   S   K   D   S   T   Y   S   L   S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C   E   V   T   H   Q
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FIG. 3-2

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1101  GGCCTGAGCT CGCCCGTCAC AAAGAGCTTC AACAGGGGAG AGTGTTAAGC TGATCCTCTA CGCCGGGACGC ATCGTGGCCC TAGTACGCAA GTTCACGTAA
      CCGGACTCGA GCGGGCAGTG TTTCTCGAAG TTGTCCCCTC TCACAATTCG ACTAGGAGAT GCGGCCTGCG TAGCACCGGG ATCATGCGTT CAAGTGCATT
 222    G   L   S     P   V   T     K   S   F     N   R   G     E   C   Q

1201  AAAGGGTATC TAGAGGTTGA GGTGATTTTA TGAAAAAGAA TATCGCATTT CTTCTTGCAT CTATGTTCGT TTTTTCTATT GCTACAAACG CGTACGCTCA
      TTTCCCATAG ATCTCCAACT CCACTAAAAT ACTTTTTCTT ATAGCGTAAA GAAGAACGTA GATACAAGCA AAAAAGATAA CGATGTTTGC GCATGCGAGT
  1                                             M   K   K   N   I   A   F     L   L   A   S   M   F   V   F   S   I   A   T   N   A   Y   A   Q

1301  GGCTTATCTG CAGCAGTCTG GGGCCGAGCT GGTGCGGCCA TCAGAGATGTC TCAAGATGTC CTGTAAAGCT CCTTCACCAG CTATAACATG
      CCGAATAGAC GTCGTCAGAC CCCGGCTCGA CCACGCCGGT AGTCTCTACAG AGTTCTACAG GACATTTCGA GGAAGTGGTC GATATTGTAC
 25     A   Y   L   Q   Q   S   G   A   E   L   V   R   P   G   A   S   V   K   M   S   C   K   A   S   G   Y   T   F   T   S   Y   N   M

1401  CATTGGGTCA AGCAGACACC GAGGCAAGGC CTGGAATGGA TTGGAGCGAT CTATCCTGGC AACGGCGACA CGAGCTATAA CCAGAAGTTC AAGGGCAAGG
      GTAACCCAGT TCGTCTGTGG CTCCGTTCCG GACCTTACCT AACCTCGCTA GATAGGACCG TTGCCGCTGT GCTCGATATT GGTCTTCAAG TTCCCGTTCC
 58     H   W   V   K   Q   T   P   G   Q   G   L   E   W   I   G   A   I   Y   P   G   N   G   D   T   S   Y   N   Q   K   F   K   G   K   A

1501  CCACTCTGAC TGTGGACAAG TCCAGCAGTA CTGCCTACAT GCAACTGAGC AGCCTGACTT CTGAGGACAG CGCTGTCTAC TTTTGTGCTC GCGTGGTCTA
      GGTGAGACTG ACACCTGTTC AGGTCGTCAT GACGGATGTA CGTTGACTCG TCGGACTGAA GACTCCTGTC GCGACAGATG AAAACACGAG CGCACCAGAT
 92     T   L   T   V   D   K   S   S   S   T   A   Y   M   Q   L   S   S   L   T   S   E   D   S   A   V   Y   F   C   A   R   V   V   Y

1601  CTATAGCAAC AGCTACTGGT ACTTCGACGT CTGGGGTACC GGAACCACAG TCACCGTCTC CTCGGCCTCC ACCAAGGGCC CATCGGTCTT CCCCCTGGCA
      GATATCGTTG TCGATGACCA TGAAGCTGCA GACCCCATGG CCTTGGTGTC AGTGGCAGAG GAGCCGGAGG TGGTTCCCGG GTAGCCAGAA GGGGGACCGT
125     Y   S   N   S   Y   W   Y   F   D   V   W   G   T   G   T   T   V   T   V   S   S   A   S   T   K   G   P   S   V   F   P   L   A

1701  CCCTCCTCCA AGAGCACCTC TGGGGGCACA GCGGCCCTGG GCTGCCTGGT CAAGGACTAC TTCCCCGAAC CGGTGACGGT GTCGTGGAAC TCAGGCGCCC
      GGGAGGAGGT TCTCGTGGAG ACCCCCGTGT CGCCGGGACC CGACGGACCA GTTCCTGATG AAGGGGCTTG GCCACTGCCA CAGCACCTTG AGTCCGCGGG
158     P   S   S   K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L

1801  TGACCAGCGG CGTGCACACC TTCCCGGCTG TCCTACAGTC CTCAGGACTC TACTCCCTCA GCAGCGTGGT GACCGTGCCC TGGGCCACCCA TCAGGAGCGT
      ACTGGTCGCC GCACGTGTGG AAGGGCCGAC AGGATGTCAG GAGTCCTGAG ATGAGGGAGT CGTCGCACCA CTGGCACGGG ACCCGGTGGT AGTCCTCGCA
192     T   S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y   S   L   S   S   V   V   T   V   P   S   S   S   L   G   T   Q

1901  GACCTACATC TGCAACGTGA ATCACAAGCC CAGCAACACC AAGGTGGACA AGAAAGTTGA GCCCAAATCT TGTGACAAAA CTCACACATG ACCACCGCAT
      CTGGATGTAG ACGTTGCACT TAGTGTTCGG GTCGTTGTGG TTCCACCTGT TCTTTCAACT CGGGTTTAGA ACACTGTTTT GAGTGTGTAC TGGTGGCGTA
225     T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D   K   K   V   E   P   K   S   C   D   K   T   H   T   Q

2001  GCACCAGTAT CGTCCATTCC GACAGCATCG CCAGTCACTA TGGCGTGCTG CCAGCAGCTC CGCTGCCTCC CCTATACCTT GTCTGCCTCC CCGGCGTTGCA
      CGTGGTCATA GCAGGTAAGG CTGTCGTAGC GGTCAGTGAT ACCGCACGAC GGTCGTCGAG GCGACGGAGG GGATATGGAA CAGACGGAGG GGCCGCAACGC

2101  TGGAGCCGGG CCACCTCGAC CTGAATGGAA CTGAACTCACCA GGATTCACCA CCTCGCTAAC TGGAGCCAAT CAATTCTTGC GGAGAACTGT
      ACCTCGGCCC GGTGGAGCTG GACTTACCTT GACTTGAGTGGT CCTAAGTGGT GGAGCGATTG ACCTCGGTTA GTTAAGAACG CCTCTTGACA

2201  GAATGCGCAA ACCAACCCTT GGCAGAACAT ATCCATGCGG TCCGCCATCT CCAGCAGCGC CACGCGGCGC ATCTCGGCGC CTGGCCACGG CTGGCCGGTGCC
      CTTACGCGTT TGGTTGGGAA CCGTCTTGTA TAGGTAGCGC AGGCGGTAGA GGTCGTCGCG GTGCGCCGCG TAGAGCCGCG GACCGGTGCC GACCGGCGCC
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FIG. 3-3
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2301  GTGCGCATGA  TCGTGCTCCT  GTCGTTGAGG  ACCCGGCTAG  GCTGGCGGGG  TTGCCTTACT  GGTTAGCAGA  ATGAATCACC  GATACGCGAG  CGAACGTGAA
      CACGCGTACT  AGCACGAGGA  CAGCAACTCC  TGGGCGCCCC  CGACCGCCCC  AACGGAATGA  CCAATCGTCT  TACTTAGTGG  CTATGCGCTC  GCTTGCCACTT

2401  GCGACTGCTG  CTGCAAAACG  TCTGCGACCT  GAGCAACAAC  ATGAAGGTTC  TTCGGTTTCC  GTGTTTCGTA  AGTCTGGAA   ACGCGGAAGT  CAGCGCCCTG
      CGCTGACGAC  GACGTTTGC   GACGCTGGA   CTCGTTGTTG  TACTTACCAG  AAGCCAAAGG  CACACAAGCAT  TTCAGACCTT  TGCGCCTTCA  GTCGCGGGAC

2501  CACCATTATG  TTCCGGATCT  GCATCGCAGG  ATGTGCTGG   CTACCCTGTG  GAACACCTAC  ATCTGTATTA  ACGAAGCGCT  GGCATTGACC  CTGAGTGATT
      GTGGTAATAC  AAGGCCTAGA  CGTAGCGTCC  TACGACGACC  GATGGGATG   CTTGTGGATG  TAGACATAAT  TGCTTCGCGA  CCGTAACTGG  GACTCACTAA

2601  TTTCTCTGGT  CCCGCCGCAT  CCATACCGCC  AGTTGTTTAC  CCTCACAACG  TTCCAGTAAC  CATCATCAGT  AACCCGTATC  GTGAGCATCC
      AAAGAGACCA  GGGCGGCGTA  GGTATGGCGG  TCAACAAATG  GGAGTGTTGC  AAGGTCATTG  GTAGTAGTCA  TTGGGCATAG  CACTCGTAGG

2701  TCTCTCGTTT  CATCGGTATC  ATTACCCCCA  TGAACAGAAA  TTCCCCCCTTA  CACGGAGGCA  TCAAGTGACC  AAACAGGAAA  AAACCGCCCT  TAACATGGCC
      AGAGAGCAAA  GTAGCCATAG  TAATGGGGGT  ACTTGTCTTT  AAGGGGGAAT  GTGCCTCCGT  AGTTGCTTCGGA  TTTGTCCTTT  TTTGGCGGGA  ATTGTACCGG

2801  CGCTTTATCA  GAAGCCAGAC  ATTAACGCTT  CTGGAGAAAC  TCAACGAGCT  GGACGCGGAT  ACATCTGTGA  ATCGCTTCAC  GACCACGCTG
      GCGAAATAGT  CTTCGGTCTG  TAATTGCGAA  GACCTCTTTG  AGTTGCTCGA  CCTGCGCCTA  TGTAGACACT  TAGCGAAGTG  CTGGTGCGAC

2901  ATGGCGGTTTA  CCGCAGCATC  CGGAAATTGT  AAATTTTTGT  TAAATTCGCGTT  AAATTTTTAA  CCAATAGGCC
      TACTGCGAAAT  GGCGTCGTAG  GCCTTTAACA  TTTAAAAACA  ATTTAAGCGCAA  TTTAAAAATT  GGTTATCCGG

3001  GAAATCGGCA  AAATCCCTTA  TAAATCAAAA  GAATAGACCG  AGATAGGGTT  GAGTGTTGTT  ACAAGAGTCC  ACTATTAAAG  AACGTGGACT
      CTTTAGCCGT  TTTAGGGAAT  ATTTAGTTTT  CTTATCTGGC  TCTATCCCAA  CTCACAACAA  GGTCAAACCT  TGATAATTTC  TTGCACCTGA

3101  CCAACGTCAA  AGGGGCGAAAA  ACCGTCTATC  AGGGCTATGG  CCCACTACGT  GAACCATCAC  CCTAATCAAG  TTTTTTGGGG  TCGAGGTGCC  GTAAAGCACT
      GGTTGCAGTT  TCCCGCTTTT  TGGCAGATAG  TCCCGATACC  GGGTGATGCA  CTTGGTAGTG  GGATTAGTTC  AAAAAACCCC  AGCTCCACGG  CATTTCGTGA

3201  AAATCGGAAC  CCTAAAGGGA  GCCCCCCGATT  TAGAGCTTGA, CGGGGAAAGC  CGGCGAACGT  GGCGAGAAAG  GAAGGGAAGA  AAGCGAAAGG ,AGCGGGCGCT
      TTTAGCCTTG  GGATTTCCCT  CGGGGCTAA   ATCTCGAACT  GCCCCTTTCG  GCCGCTTGCA  CCGCTCTTTCT  CTTCCCTTCT  TTCGCTTTCC  TCGCCCGCGA

3301  AGGGGCGCTGG  CAAGTGGTAGC  GGTCACGCTG  CGCGTAACCA  CCACACCCGC  CGGCGCTTAAT  GCGGCGACGT  AGGGCGCGTC  CGCATCCTGC  CTCGCGCGTT
      TCCCGCGACC  GTTCACATCG  CCAGTGCGAC  GCGCATTGGT  GGTGTGGGCG  GCCGCGAATTA  CGCGCTGGCA  TCCCGCGCAG  GCGTAGGAGG  GAGCGCGCAA

3401  TCGGTGATGA  CGGTGAAAAC  CTCTGACACA  TGCAGCTCCC  CCGCTAACCA  ACAGCTTGTC  TGTAAGCGGA  AGACAAGCCC  GTCAGGGCGC
      AGCCACTACT  GCCACTTTTG  GAGACTGTGT  ACGTCGAGGG  GGCGGCGAATTA  TGTCGAACAG  ACATTCGCCT  ACGGCCCTCG  TCTGTTCGGG  CAGTCCCGCG

3501  GTCAGCGGGGT  GTTGGCGGGT  GTCGGGGCGC  AGCCATGACC  CAGTCACGTA  GCGATAGCGG  AGTGTATACT  GGCTTAACTA  TGCGGCATCA  GAGCAGATTG
      CAGTCGCCCA  CAACGCCCCA  CAGCCCCGCG  TCGGTACTGG  GTCAGTGCAT  CGCTATCGCC  TCACATATGA  CCGAATTGAT  ACGCCGTAGT  CTCGTCTAAC

3601  TACTGAGAGT  GCACCATATG  CGGTGTGAAA  TACCGCCACAG  ATGCCTAAGG  AGAAAATACC  CTCTTCCGCT  CCTCGCGCT   CTGACTCGCT
      ATGACTCTCA  CGTGGTATAC  GCCACACTTT  ATGGCGGTGTC  TACGGCATTCC  TCTTTTATGG  GAGAAGGCGA  AGGAGCGAGT  GACTGAGCGA

3701  GCGCTCGGTC  GTTCGGCTGC  GGCGAGCGGT  ATCAGCTCAC  TCAAAGGCGG  TAATACGGTT  ATCCACAGAA  TCAGGGGATA  ACGCAGGAAA  GAACATGTGA
      CGCGAGCCAG  CAAGCCGACG  CCGCTCGCCA  TAGTCGAGTG  AGTTTCCGCC  ATTATGCCAA  TAGGTGTCTT  AGTCCCCTAT  TGCGTCCTTT  CTTGTACACT
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FIG. 3-4

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3801  GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT
      CGTTTTCCGG TCGTTTTCCG GTCCTTGGCA TTTTTCCGGC GCAACGACCG CAAAAAGGTA TCCGAGGCGG GGGGACTGCT CGTAGTGTTT TTAGCTGCGA

3901  CAAGTCAGAG GTGGCGAAAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GTTCCGACCC TGCCGCTTAC
      GTTCAGTCTC CACCGCTTTG ATATTTCTAT GGTCCGCAAA GGGGGACCTT CGAGGGAGCA CAAGGCTGGG ACGGCGAATG

4001  CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT CTCCAAGCTG
      GCCTATGGAC AGGCGGAAAG AGGGCGAAAG TTCGCCACCG GAAAGAGTAT CGAGTGCGAC TCAAGCCACA TCCAGCAAGC GAGGTTCGAC

4101  GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG
      CCGACACACG TGCTTGGGGG GCAAGTCGGG CTGGCGACGC GGAATAGGCC ATTGATAGCA GAACTCAGGT TGGGCCATTC TGTGCTGAAT AGCGGTGACC

4201  CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT
      GTCGTCGGTG ACCATTGTCC TAATCGTCTC GCTCCATACA TCCGCCACGA TGTCTCAAGA ACTTCACCAC CGGATTGATG CCGATGTGAT CTTCCTGTCA

4301  ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT
      TAAACCATAG ACGCGAGACG ACTTCGGTCA ATGGAAGCCT TTTTCTCAAC CATCGAGAAC TAGGCCGTTT GTTTGGTGGC GACCATCGCC ACCAAAAAAA

4401  GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT
      CAAACGTTCG TCGTCTAATG CGCGTCTTTT TTCCTAGAG TTCTTCTAGG AAACTAGAAA AGATGCCCCA GACTGCGAGT CACCTTGCTT TTGAGTGCAA

4501  AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC
      TTCCCTAAAA CCAGTACTCT AATAGTTTTT CCTAGAAGTG GATCTAGGAA AATTTAATTT TTACTTCAAA ATTTAGTTAG ATTTCATATA TACTCATTTG

4601  TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA
      AACCAGACTG TCAATGGTTA CGAATTAGTC ACTCCGTGGA TAGAGTCGCT AGACAGATAA AGCAAGTAGG TATCAACGGA CTGAGGGGCA GCACATCTAT

4701  ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG
      TGATGCTATG CCCTCCCGAA TGGTAGACCG GGGTCACGAC GTTACTATGG CGCTCTGGGT GCGAGTGGCC GAGGTCTAAA TAGTCGTTAT TTGGTCGGTC

4801  CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA
      GGCCTTCCCG GCTCGCGTCT TCACCAGGAC GTTGAAATAG GCGGAGGTAG GTCAGATAAT TAACAACGGC CCTTCGATCT CATTCATCAA GCGGTCAATT

4901  TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA
      ATCAAACGCG TTGCAACAAC GGTAACGACG TCCGTAGCAC CACAGTGCGA GCAGCAAACC ATACCGAAGT AAGTCGAGGC CAAGGGTTGC TAGTTCCGCT

5001  GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA
      CAATGTACTA GGGGGTACAA CACGTTTTTT CGCCAATCGA GGAAGCCAGG AGGCTAGCAA CAGTCTTCAT TCAACCGGCG TCACAATAGT GAGTACCAAT

5101  TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG
      ACCGTCGTGA CGTATTAAGA GAATGACAGT ACGGTAGGCA TTCTACGAAA AGACACTGAC CACTCATGAG TTGGTTCAGT AAGACTCTTA TCACATACGC

5201  GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA
      CGCTGGCTCA ACGAGAACGG GCCGCAGTTG TGCCCTATTA TGGCGCGGTG TATCGTCTTG AAATTTTCAC GAGTAGTAAC CTTTTGCAAG AAGCCCCGCT
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FIG. 3-5
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5301  AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG
      TTTGAGAGTT CCTAGAATGG CGACAACTCT AGGTCAAGCT ACATTGGGTG AGCACGTGGG TTGACTAGAA GTCGTAGAAA ATGAAAGTGG TCGCAAAGAC

5401  GGTGAGCAAA AACAGGAAGG CAAAAATGCCG AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG
      CCACTCGTTT TTGTCCTTCC GTTTTTACGGC GTTTTTCCC TTATTCCCGC TGTGCCTTTA CAACTTATGA GTATGAGAAG GAAAAAGTTA TAATAACTTC

5501  CATTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTCCCCGCA AAAAGTGCCA
      GTAAATAGTC CCAATAACAG AGTACTCGCC TATGTATAAA CTTACATAAA TCTTTTATT TGTTTATCCC CAAGGCGCGT TTTTCACGGT

5601  CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTTCAA
      GGACTGCAGA TTCTTTGGTA ATAATAGTAC TGTAATTGGA TATTTTATC CGCATAGTGC TCCGGGAAAG CAGAAGTT
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FIG. 4 - plasmid pDR1

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TTCGAGCTCGCCCGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCA
TTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCT
GGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTA
ACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCAC
TTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGT
AAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAG
TACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAAT
GGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAAT
GGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCC
CCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGAGGTCTATATAAGCAGAGCTCGT
TTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGA
CACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGT
GCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCTTCGTTA
GAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACAC
TATAGAATAACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGTCCAACTGC
ACCTCGGTTCTATCGATTGAATTCCACCATGGGATGGTCATGTATCATCCTTTTTCTAGT
AGCAACTGCAACTGGAGTACATTCAGATATCCAGATGACCCAGTCCCCGAGCTCCCTGTC
CGCCTCTGTGGGCGATAGGGTCACCATCACCTGCCGTGCCAGTCAGGACATCCGTAATTA
TTTGAACTGGTATCAACAGAAACCAGGAAAAGCTCCGAAACTACTGATTTACTATACCTC
CCGCCTGGAGTCTGGAGTCCCTTCTCGCTTCTCTGGTTCTGGTTCTGGGACGGATTACAC
TCTGACCATCAGTAGTCTGCAACCGGAGGACTTCGCAACTTATTACTGTCAGCAAGGTAA
TACTCTGCCGTGGACGTTCGGACAGGGCACCAAGGTGGAGATCAAACGAACTGTGGCTGC
ACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGT
TGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAA
CGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCAC
CTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTA
CGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGG
AGAGTGTTAAGCTTGGCCGCCATGGCCCAACTTGTTTATTGCAGCTTATAATGGTTACAA
ATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTG
TGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCGATCGGGAATTAATTCG
GCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGGTACCTTCTGAG
GCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCC
CAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGT
CCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCA
TAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTC
CGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTG
AGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTGT
TAACAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTAC
CCAACTTAATCGCCTTGCAGCACATCCCCCCTTCGCCAGCTGGCGTAATAGCGAAGAGGC
CCGCACCGATCGCCCTTCCCAACAGTTGCGTAGCCTGAATGGCGAATGGCGCCTGATGCG
GTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATACGTCAAAGCAACCATAG
TACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACC
GCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCC
ACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTT
AGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGG
CCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGT
GGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTA
TAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTT
AACGCGAATTTTAACAAAATATTAACGTTTACAATTTTATGGTGCACTCTCAGTACAATC
TGCTCTGATGCCGCATAGTTAAGCCAACTCCGCTATCGCTACGTGACTGGGTCATGGCTG
CGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCAT
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FIG. 4 - cont'd

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CCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGT
CATCACCGAAACGCGCGAGGCAGTATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTAT
TTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGG
GAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
TCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTA
TTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTG
CTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGG
GTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAAC
GTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGATG
ACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT
ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTG
CTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGAC
CGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTT
GGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCAGCAG
CAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGC
AACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCC
TTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTA
TCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGG
GGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGA
TTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAAC
TTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAA
TCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGAT
CTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGC
TACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTG
GCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACC
ACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGG
CTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGG
ATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAA
CGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCG
AAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGA
GGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCT
GACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCA
GCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTC
CTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCG
CTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCC
CAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATCCAGCTGGCACGACA
GGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTACCTCACTC
ATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGA
GCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTAA
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FIG. 5 - plasmid pDR2

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ATTCGAGCTCGCCCGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTC
ATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCC
TGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGT
AACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCA
CTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGG
TAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCA
GTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAA
TGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGC
CCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCG
TTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAG
ACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCG
TGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCTTCGTT
AGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACATACGATTTAGGTGACA
CTATAGAATAACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGTCCAACTG
CACCTCGGTTCTATCGATTGAATTCCACC̲A̲T̲G̲GGATGGTCATGTATCATCCTTTTTCTAG
TAGCAACTGCAACTGGAGTACATTCAGAAGTTCAGCTGGTGGAGTCTGGCGGTGGCCTGG
TGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGCAGCTTCTGGCTACTCCTTTACCGGCT
ACACTATGAACTGGGTGCGTCAGGCCCCAGGTAAGGGCCTGGAATGGGTTGCACTGATTA
ATCCTTATAAAGGTGTTACTACCTATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCG
TAGATAAATCCAAAAACACAGCCTACCTGCAAATGAACAGCCTGCGTGCTGAGGACACTG
CCGTCTATTATTGTGCTAGAAGCGGATACTACGGCGATAGCGACTGGTATTTTGACGTCT
GGGGTCAAGGAACCCTGGTCACCGTCTCCTCGGCCTCCACCAAGGGCCCATCGGTCTTCC
CCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCA
AGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCG
TGCACACCTTCCCGGCTGTCCTAC̲AGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGA
CTGTGCCCTCTAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCA
GCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAAACTCACACATGCC
CACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAAC
CCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGA
GCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG
CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCA
CCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAG
CCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCAC
AGGTGTACACCCTGCCCCCATCCCGGGAAGAGATGACCAAGAACCAGGTCAGCCTGACCT
GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGC
CGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCT
ACAGCAAGCTCACCGTGGACAAGAGC̱AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCG
TGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA
AA̲T̲G̲A̲GTGCGACGGCCCTAGAGTCGACCTGCAGAAGCTTGGCCGCCATGGCCCAACTTGT
TTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAG
CATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATG
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GGAACTTGGTTAGGTACCTTCTGAGGCGGAAAGAACCATCTGTGGAATGTGTGTCAGTTA
GGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAAT
TAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGC
ATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTA
ACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCA
GAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGA
GGCCTAGGCTTTTGCAAAAAGCTGTTAACAGCTTGGCACTGGCCGTCGTTTTACAACGTC
GTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCCTTCG
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FIG. 5 Cont.'d

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CCAGTTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGTAGCC
TGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCAC
ACCGCATACGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGG
TGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTT
CGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCG
GGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGA
TTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGAC
GTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCC
TATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAA
AAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAAT
TTTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAACTCCGCTA
TCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCC
TGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGC
TGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGTATTCTTGAAGA
CGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCT
TAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTC
TAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAA
TATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTT
GCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCT
GAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATC
CTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTA
TGTGGCGCGGTATTATCCCGTGATGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACAC
TATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGC
ATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAAC
TTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGG
GATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGAC
GAGCGTGACACCACGATGCCAGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGC
GAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTT
GCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGA
GCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCC
CGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAG
ATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCA
TATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCA
GACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGC
TGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTA
CCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTT
CTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTC
GCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGG
TTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCG
TGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAG
CATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC
AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTAT
AGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGG
GGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGC
TGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATT
ACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCA
GTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCG
ATTCATTAATCCAACTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAAC
GCAATTAATGTGAGTTACCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG
GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGAC
CATGATTACGAATTA
```

FIG. 6

hu2H7.v16 L chain [232 aa]

MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGK
APKPLIYAPSNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFGQGT
KVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES
VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 7

hu2H7.v16 H chain　　[471 aa]

MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAP
GKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVVY
YSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FIG. 8

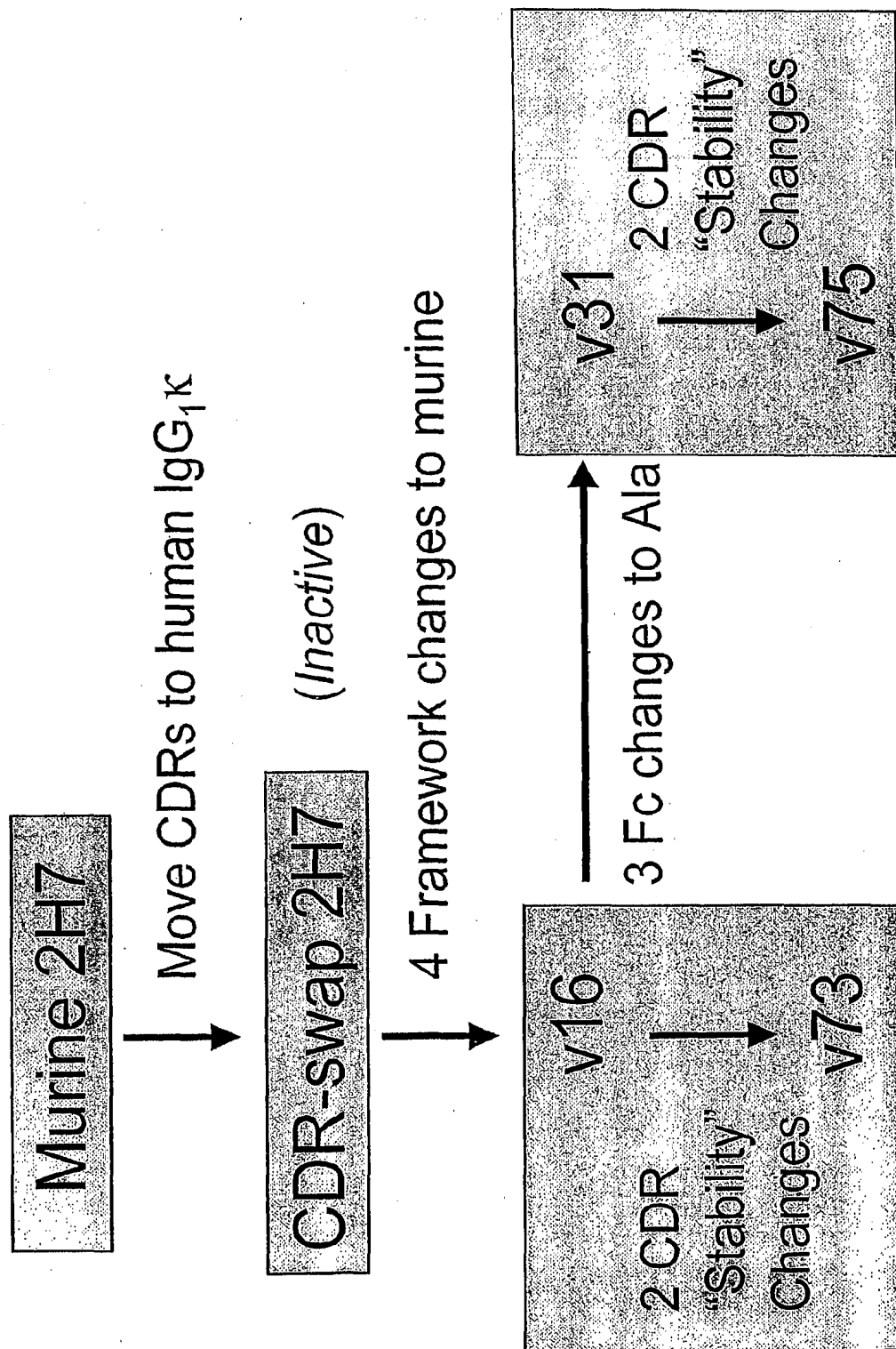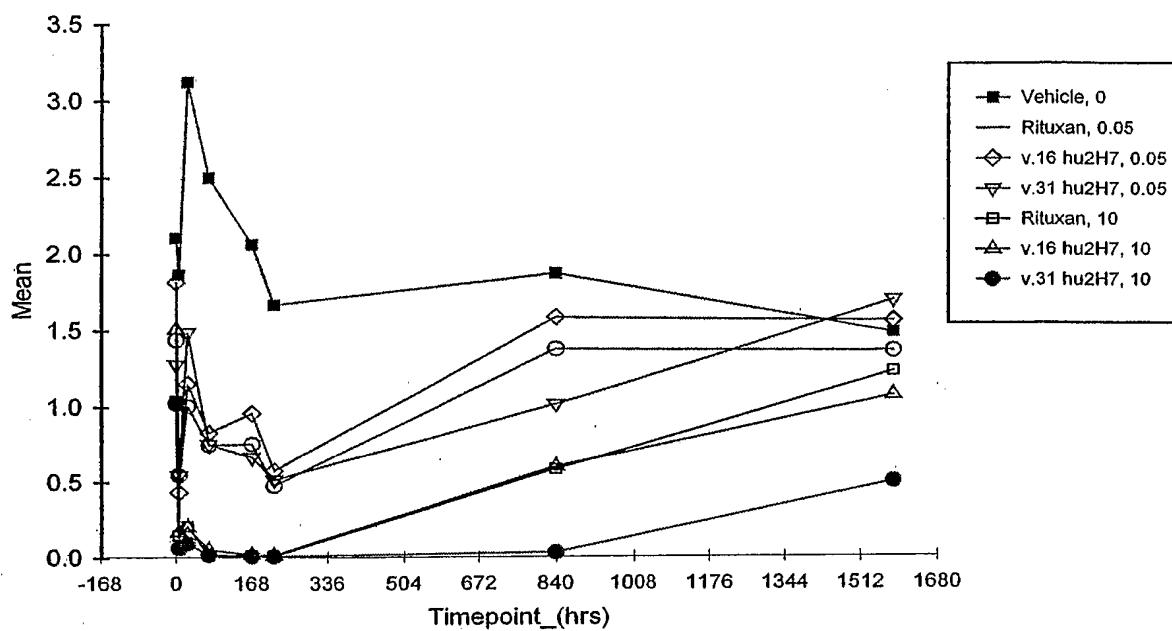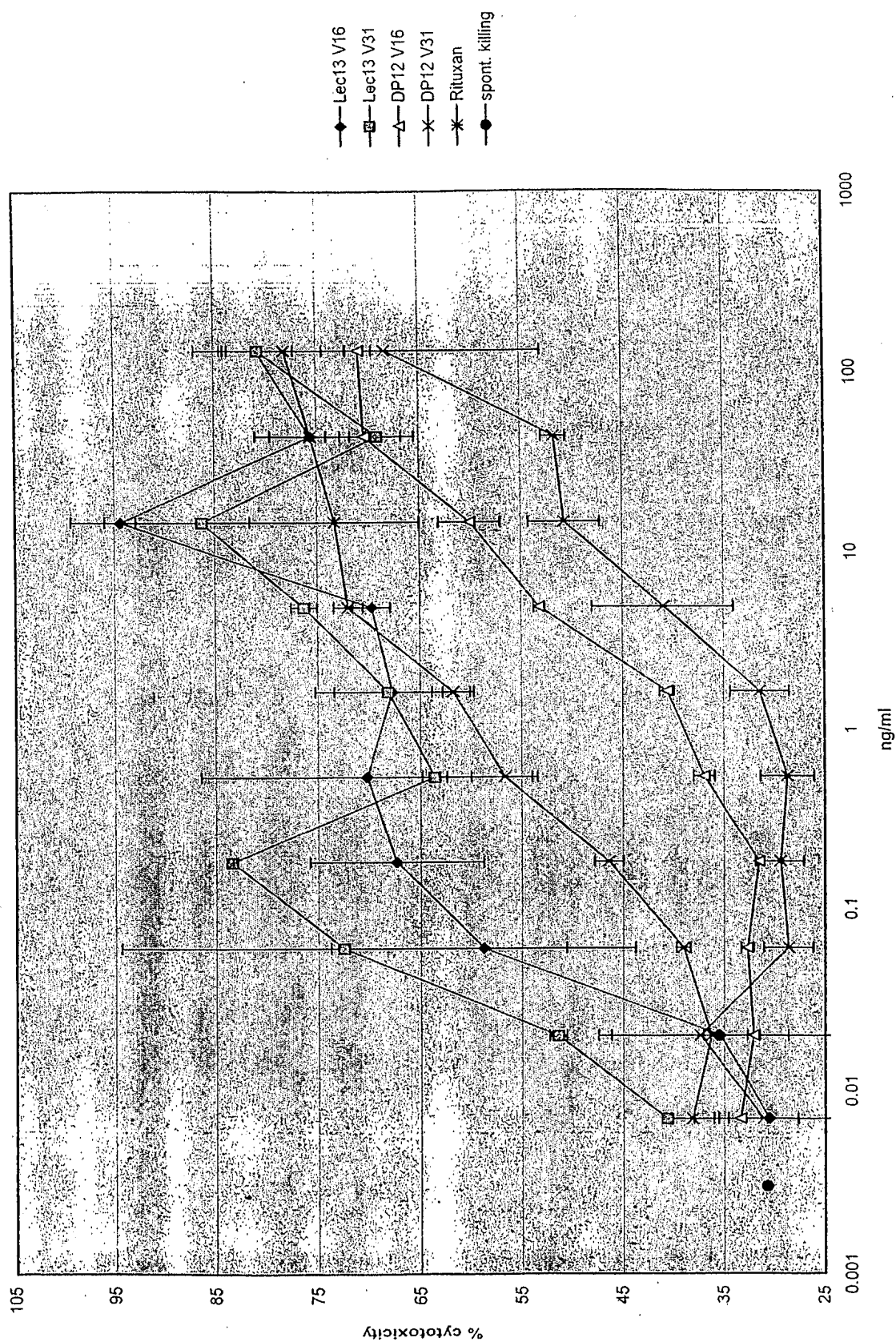hu2H7.v31_H chain [471 aa]

MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAP
GKGLEWVGAIYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVVY
YSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FIG. 9

Stability of hu2H7 versions 16 and 73
t=16 days 40°C
10 mM Histidine, 6% sucrose,
0.02% T20 pH 5.8
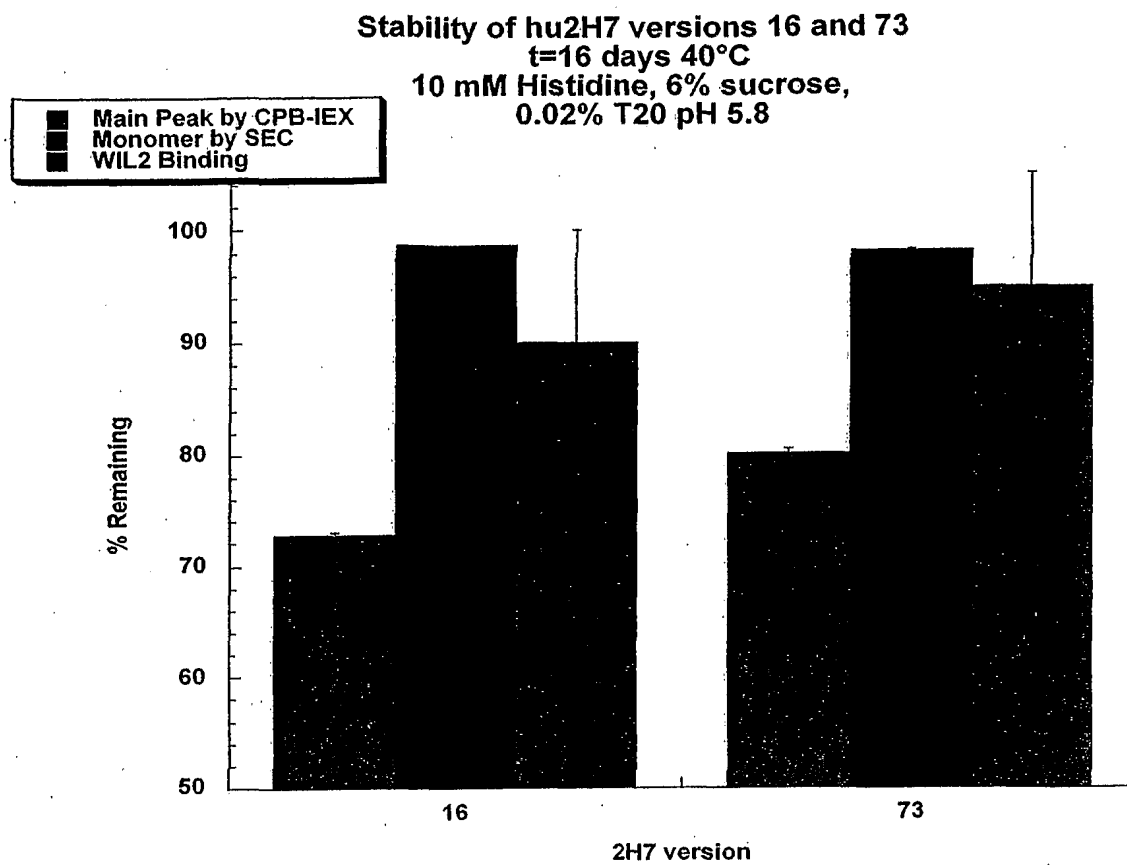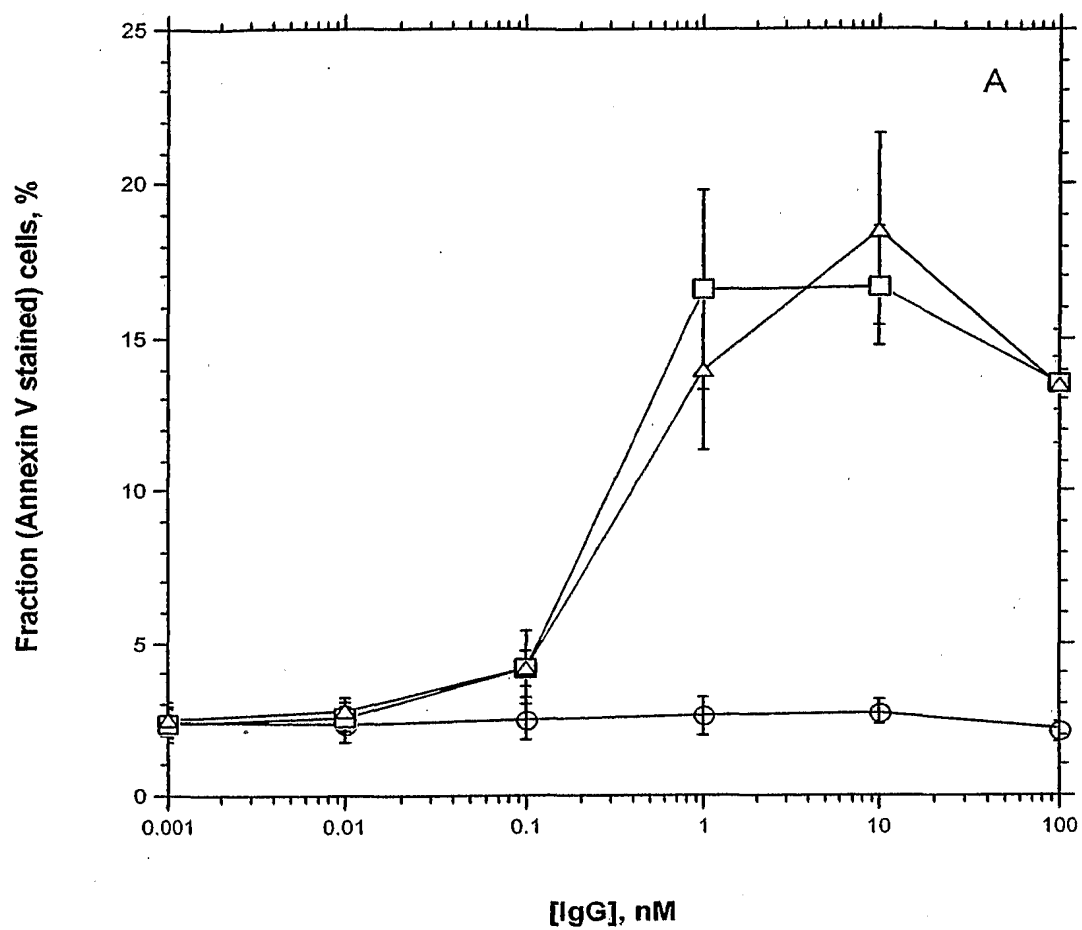
FIG. 10

FIG. 11

FIG. 12

FIG. 13

Apoptotic Activity: Annexin V Staining

FIG. 14

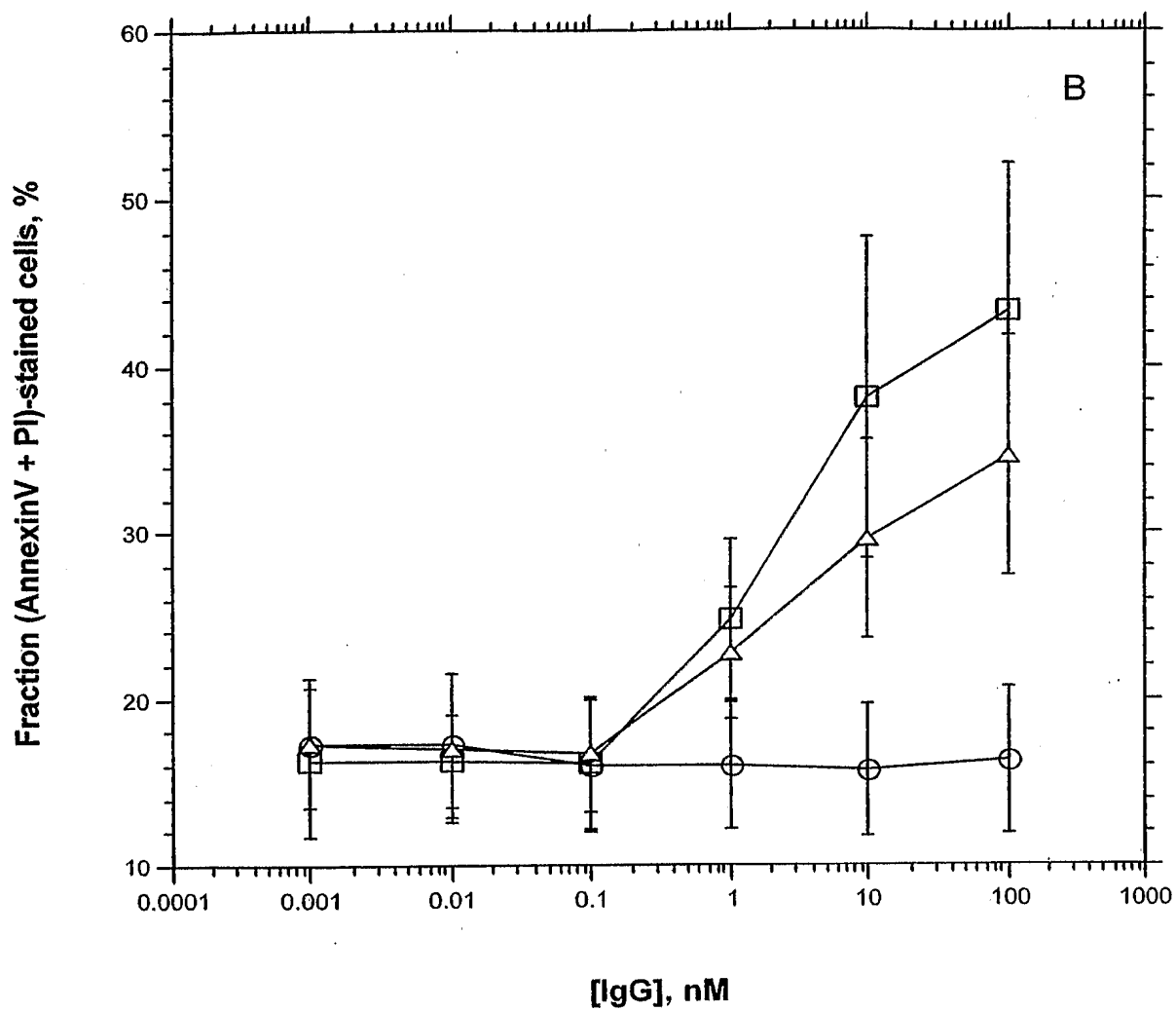Apoptotic Activity: Annexin V and Propidium Iodide Double-Staining

FIG. 15

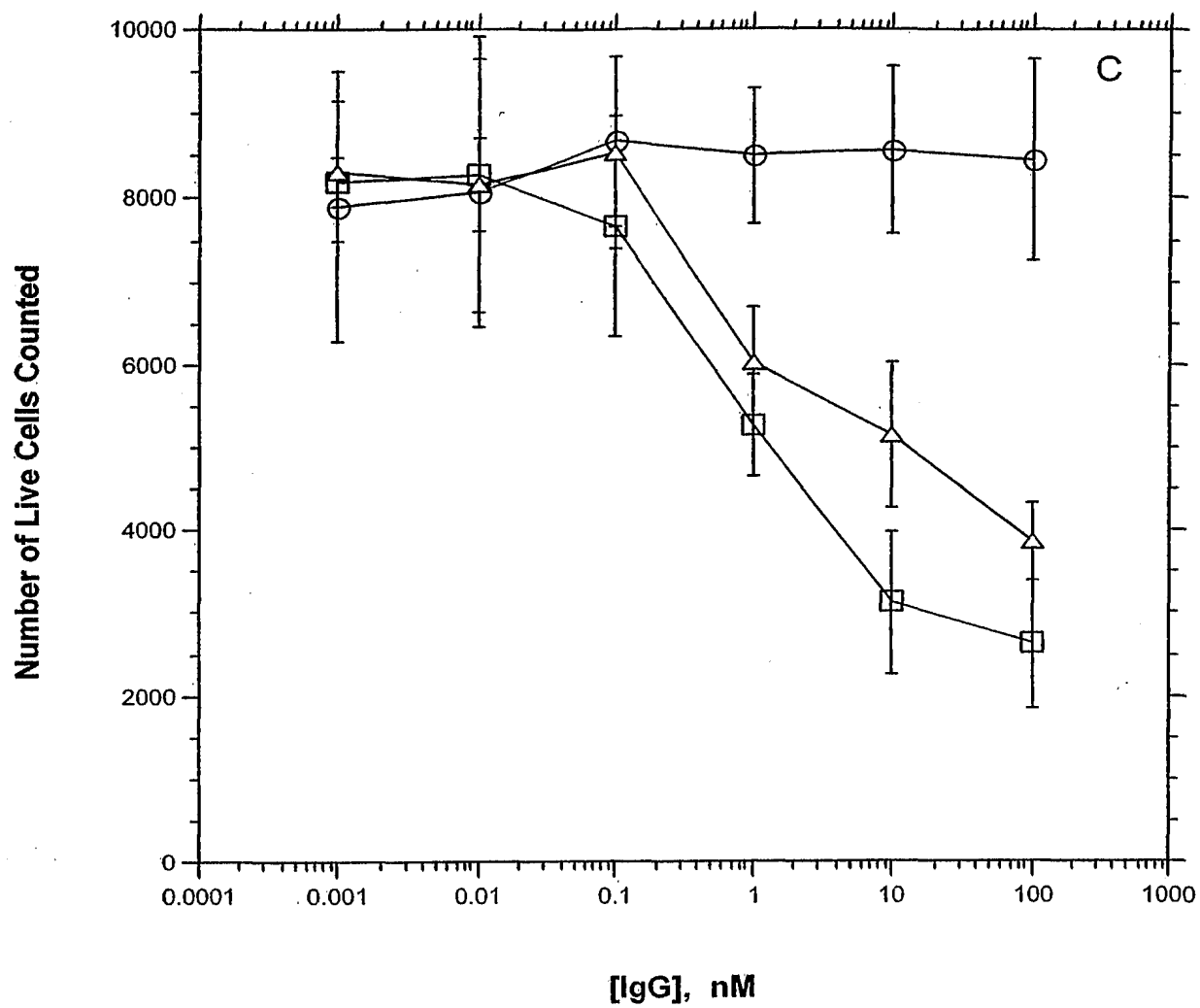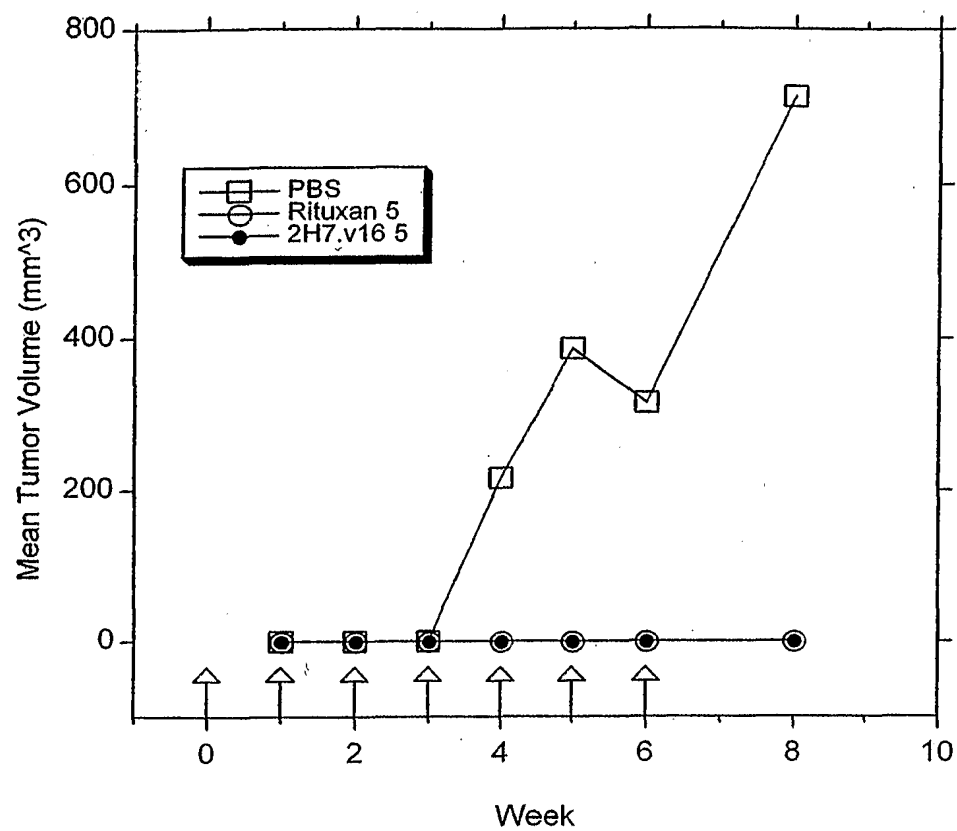## Apoptotic Activity: Live Unstained Cells

FIG. 16

FIG. 17

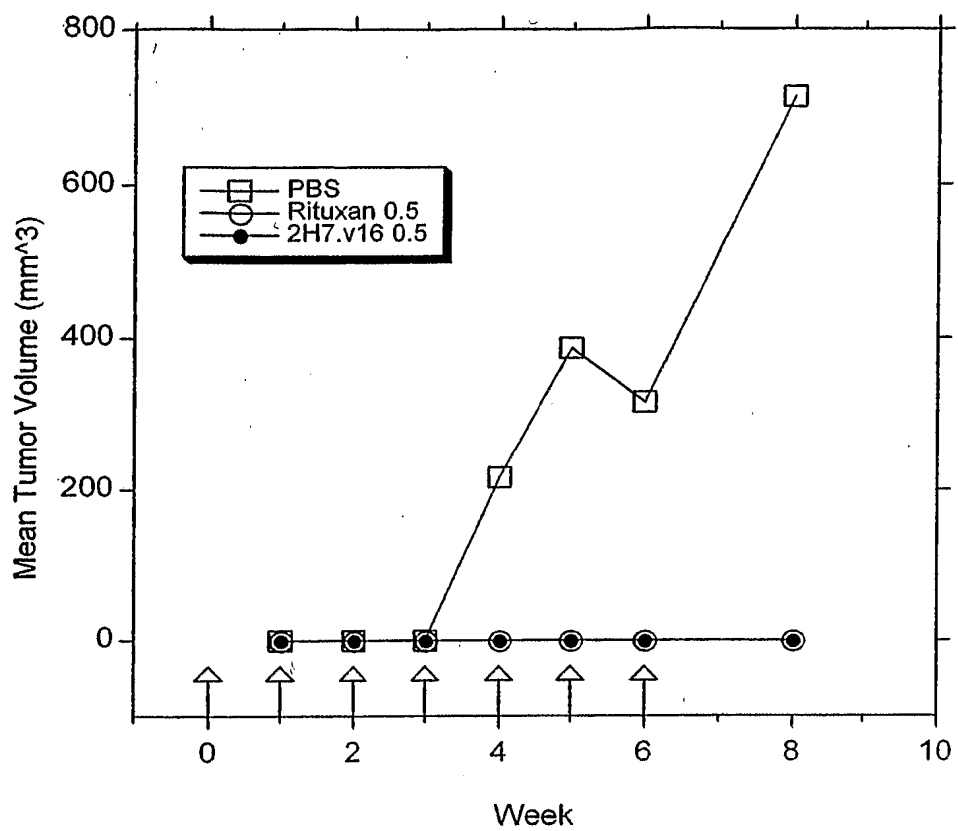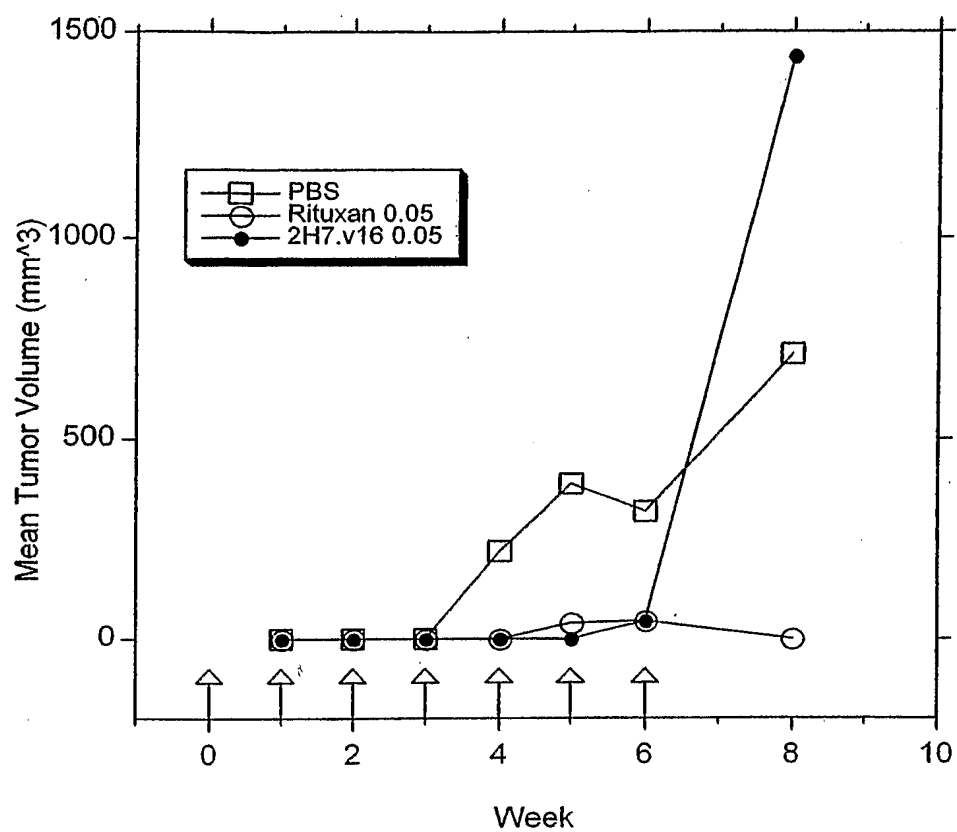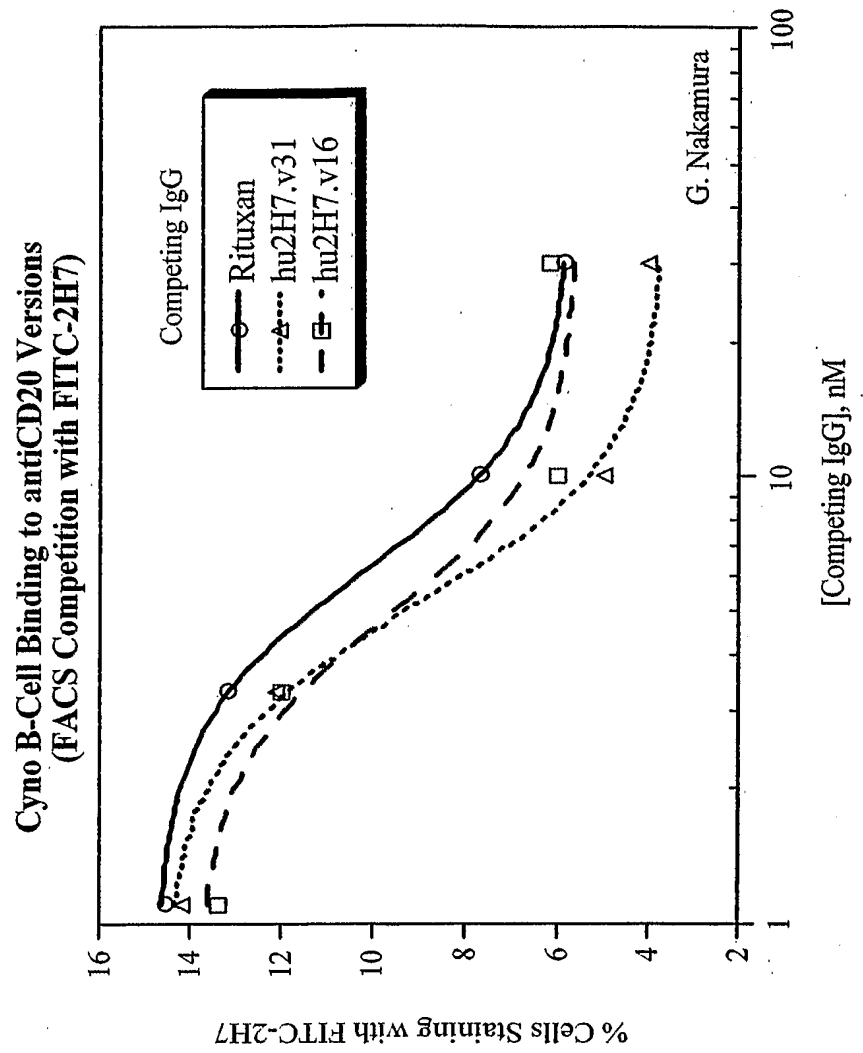FIG. 18

FIG. 19

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1    ATGACAACAC CCAGAAATTC AGTAAATGGG ACTTTCCCAG
     METThrThrPro ArgAsnSer ValAsnGly ThrPheProA

41   CAGAGCCAAT GAAAGGCCCT ATTGCTATGC AACCTGGTCC
     laGluProMET LysGlyPro IleAlaMETGln ProGlyPr

81   AAAACCACTC CTCAGGAGGA TGTCTTCACT GGTGGGTCCC
     oLysProLeu LeuArgArgMET SerSerLeu ValGlyPro

121  ACGCAAAGCT TCTTCATGAG GGAATCTAAG GCTTTGGGGG
     ThrGlnSerPhe PheMETArg GluSerLys AlaLeuGlyA

161  CTGTCCAGAT TATGAATGGG CTCTTCCACA TTGCCCTGGG
     laValGlnIle METAsnGly LeuPheHisIle AlaLeuGl

201  GGGTCTTCTG ATGATCCCAG CAGGGATCTA TGCACCCATC
     yGlyLeuLeu METIleProAla GlyIleTyr AlaProIle

241  TGTGTGACTG TGTGGTACCC TCTGTGGGGA GGCATTATGT
     CysValThrVal TrpTyrPro LeuTrpGly GlyIleMETT

281  ATATTATTTC CGGATCACTC CTGGCAGCAA CGGAGAAAAA
     yrIleIleSer GlySerLeu LeuAlaAlaThr GluLysAs

321  CTCCAGGAAG TGTTTGGTCA AAGGAAAAAT GATAATGAAT
     nSerArgLys CysLeuValLys GlyLysMET IleMETAsn

361  TCATTGAGCC TCTTTGCTGC CATTTCTGGA ATGATTCTTT
     SerLeuSerLeu PheAlaAla IleSerGly METIleLeuS

401  CAATCATGGA CATACTTAAT ATTAAAATTT CCCATTTTTT
     erIleMETAsp IleLeuAsn IleLysIleSer HisPheLe

441  AAAAATGGAG AGTCTGAATT TTATCAGAGT TCACACACCA
     uLysMETGlu SerLeuAsnPhe IleArgVal HisThrPro

481  TATATTAACA TATACAACTG TGAACCAGCT AATCCCTCTG
     TyrIleAsnIle TyrAsnCys GluProAla AsnProSerG

521  AGAAAAACTC TCCATCTACT CAATACTGTT ACAGCATACA
     luLysAsnSer ProSerThr GlnTyrCysTyr SerIleGl

561  ATCTCTGTTC CTGGGCATTT TGTCAGTGAT GCTGATCTTT
     nSerLeuPhe LeuGlyIleLeu SerValMET LeuIlePhe

601  GCCTTCTTCC AGGAACTTGT AATAGCTGGC ATCGTTGAGA
     AlaPhePheGln GluLeuVal IleAlaGly IleValGluA

641  ATGAATGGAG AAGAACATGC TCCAGACCCA AATCTAGCGT
     snGluTrpArg ArgThrCys SerArgProLys SerSerVa

681  AGTTCTCCTG TCAGCTGAAG AAAAAAAAGA ACAAGTCATT
     lValLeuLeu SerAlaGluGlu LysLysGlu GlnValIle

721  GAAATAAAAG AAGAAGTGGT TGGGCTAACT GAAACATCTT
     GluIleLysGlu GluValVal GlyLeuThr GluThrSerS

761  CCCAACCAAA GAATGAAGAA GCCATTGAAA TTATTCCAAT
     erGlnProLys AsnGluGlu AlaIleGluIle IleProIl

801  CCAAGAAGAG GAAGAAGAAG AAACAGAGAC AAACTTTCCA
     eGlnGluGlu GluGluGluGlu ThrGluThr AsnPhePro

841  GAACCTCCCC AAGATCAGGA ATCTTCACCA ATAGAAAATG
     GluProProGln AspGlnGlu SerSerPro IleGluAsnA

881  ACAGCTCTCC T.
     spSerSerPro
```

FIG. 20

MTTPRNSVNGTFPAEPMKGPIAMQPGPKPLLRRMSSLVGPTQSFFMR
                          S   F

ESKALGAVQIMNGLFHIALGGLLMIPAGIYAPICVTVWYPLWGGIMYII

SGSLLAATEKNSRKCLVKGKMIMNSLSLFAAISGMILSIMDILNIKISH

FLKMESLNFIRVHTPYINIYNCEPANPSEKNSPSTQYCYSIQSLFLGILS
          A

VMLIFAFFQELVIAGIVENEWRRTCSRPKSSVVLLSAEEKKEQVIEIKE
                    K   NI   T

EVVGLTETSSQPKNEEAIEIIPIQEEEEEETETNFPEPPQDQESSPIENDS
                  D

SP

FIG. 21

Cyno B-Cell Binding to antiCD20 Versions
(FACS Competition with FITC-2H7)

Fig. 22

Dose Escalation Schema

Dose escalation will occur after treatment of 10 subjects per dose level (exception 5 subjects at Level 1).
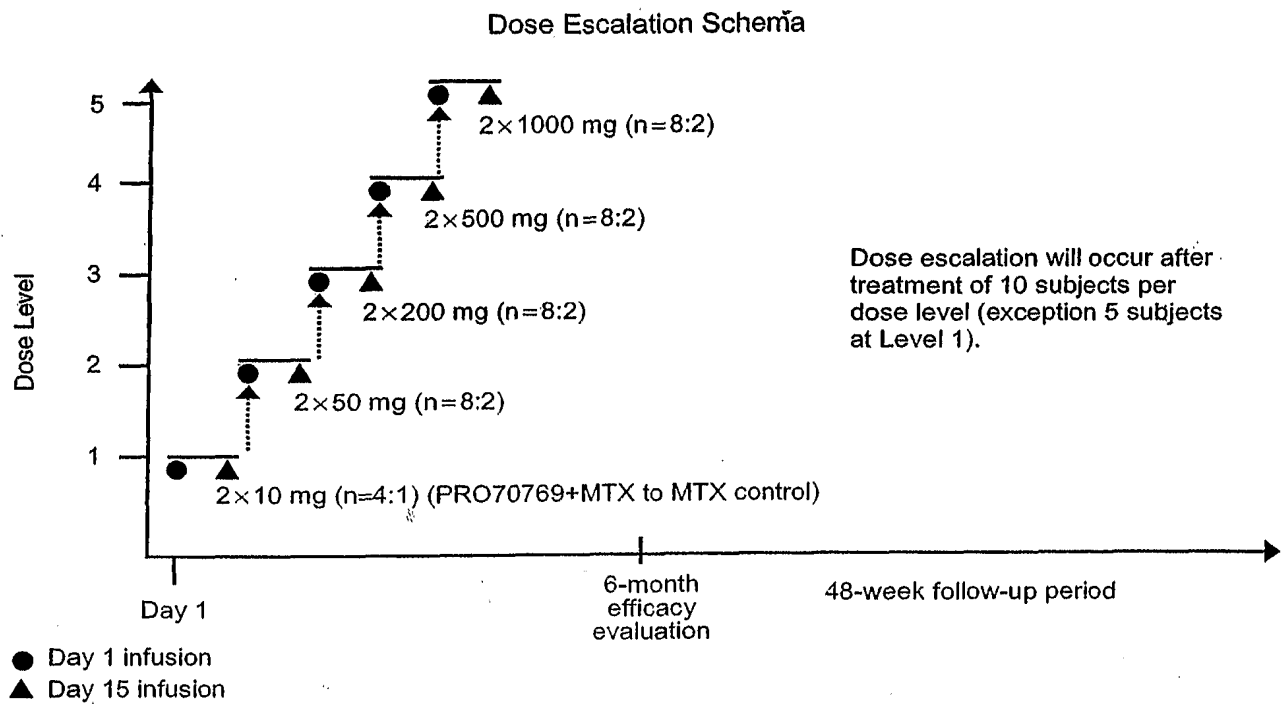
● Day 1 infusion
▲ Day 15 infusion

FIG. 23

SV40.PD.hu2H7.H16.SV.L16
8.277 kb